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ADDITIONAL NOTES ON THE ORCHIDS OF THE NEW HEBRIDES AND SANTA CRUZ ISLANDS

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In an earlier paper[1] on the orchids of the New Hebrides and Santa Cruz Islands, I included all of the species represented in S. F. Kajewski's collections with the exception of several which were too fragmentary for critical study. Since my paper was published additional material has been found and I am now able to complete the record of Kajewski's 1928 and 1929 series. During my studies I have examined a rich collection of specimens containing species that have not yet been reported to be natives of the New Hebrides or Santa Cruz group. In the following paper I have included these, and to make the record more serviceable I have included also a number of species which although already reported from the New Hebrides, are now represented by specimens recently collected or by material referable to them. Unfortunately many of the specimens found by Dr. Morrison on Aneityum lack flowers and are indeterminable. I have only included those species which are quite clear, and in a few cases, when a plant represented a genus new to the region, I have included it, although for want of flowers the specific name could not be arrived at.

For the opportunity to examine the collections made on Malekula, Efate, Eromanga, Aneityum and the Banks Islands by Dr. Morrison and L. Cheeseman, I am indebted to Sir Arthur W. Hill, Director of the Royal Botanic Gardens, Kew.

Habenaria physoplectra Reichenbach f. in Linnaea, XLI. 17 (1876).

Aneityum: Anelgauhat, *Dr. Morrison,* s. n., June 15, 1896; hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896. Efate: Undine Bay, hills, *Dr. Morrison,* s. n., August 27, 1896.

[1]Journal of the Arnold Arboretum, XIII. 127-141 (1932).

The specimens referred to this species are too old to be serviceable for purposes of accurate identification, but vegetatively they bear a close resemblance to Reichenbach's type specimen (*MacGillivray* no. **27**) and the withered remains of the flowers on the plant collected between Ithumu and Anelgauhat have the scrotiform spur that is one of the chief characters of *H. physoplectra*. MacGillivray's specimens were collected on Aneityum. Fritz Kraenzlin refers *Habenaria novoebudarum* F. v. Mueller to the synonymy of this species. (Orch. Gen. & Sp. 905).

Habenaria ponerostachys Reichenbach f. in Bonpl. III. 213 (1855).

Aneityum: Hill north of Anelgauhat, *Dr. Morrison,* s. n., June 1896.—Also Philippine Islands.

In habit and floral structure the specimen from Aneityum resembles very closely the type and specimens from Leyte and Mindanao. The broad petals, aristate lateral sepals and the lobes of the labellum are similar to corresponding structures in the Philippine plants. The extension of range is interesting, because *H. ponerostachys* is not common in the Philippine Islands and has not been reported heretofore from any other part of the world.

Habenaria stenodon Reichenbach f. in Linnaea, XLI. 17 (1876).

Banks Islands: Vanua Lava, *L. Cheeseman,* s. n., November 1929.—Already found on Aneityum by MacGillivray.

Corybas mirabilis Schlechter in Fedde, Rep. Spec. Nov. XIX. 22 (1923).

Corysanthes mirabilis Schlechter in Bull. Herb. Boiss. ser. 2, VI. 296 (1906).

Aneityum: *Dr. Morrison,* s. n., June 26, 1896 (dark glossy purple, variegated with whitish lines or meshes on lip and upper sepal).

The type was collected by Dr. Morrison on the summit of the peak between Anumy and Ithug, c. 2300 feet altitude in June 1896. The specimens in the Kew Herbarium are accompanied by a note which reads: "On peak crossed June 26, 1896."

Nervilia sp.

Aneityum: Hill north of Anelgauhat, *Dr. Morrison,* s. n., June 15, 1896.

A single leaf constitutes the specimen examined. This leaf resembles very closely the leaves of *Nervilia MacKinnonii* (Duthie) Schlechter.

Epipogon roseum (D. Don) Lindley in Jour. Linn. Soc. I. 177 (1857).

Malekula: South West Bay, in bush at sea level, *L. Cheeseman,* s. n., January 1930.—Also Java, Ceylon, New Guinea, tropical India and Australia.

Goodyera triandra Schlechter in Bull. Herb. Boiss. ser. 2, VI. 298 (1906).

Efate: Undine Bay, *Dr. Morrison,* s. n., August 20, 1896; Mount Macdonald, *Dr. Morrison,* s. n., August 18, 1896.

The specimens in the Kew Herbarium are similar to the type in being triandrous.

Platylepis Morrisonii Schlechter in Fedde, Rep. Spec. Nov. IX. 161 (1911).

Aneityum: Hills between Anelgauhat and Anumy Valley, *Dr. Morrison,* s. n., June 25, 1896; hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896.

Cystopus aneytyumensis Schlechter in Fedde, Rep. Spec. Nov. IX. 282 (1911).

Aneityum: Near summit of peak south of Ithumu, *Dr. Morrison,* s. n., June 30, 1896; ascent of peak south of Ithumu, 1000-1835 feet altitude, *Dr. Morrison,* s. n., June 30, 1896.

Cystopus fimbriatus J. J. Smith in Bull. Dép. Agric. Indes Néerl. no. 10, p. 3 (1907).

Aneityum: Hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896.—Also Dutch New Guinea.

There are minor differences between the specimens from Aneityum and the type of *C. fimbriatus,* but they are hardly sufficient to justify specific separation.

Zeuxine Erimae Schlechter in Schumann & Lauterbach, Nachtr. Fl. Deutsch. Schutzgeb. 90 (1905).

Banks Islands: Vanua Lava, Avuas, in brush, at 250 feet altitude, *L. Cheeseman,* s. n., October 7, 1929 (roots scarcely lodged in soil).—Also Kaiser-Wilhelmsland.

Anoectochilus sp.

Aneityum: Hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896 (leaves dark green, velvety dull or with sheen according to incidence of light, veined with silvery whitish lines, under surface purplish red veined with greenish).

The only specimens collected are sterile. The leaves resemble those of *Anoectochilus Roxburghii* Lindl.

Vrydagzynea Cheesemanii Ames, sp. nov.

In habitu *V. albidae* Bl. similitudinem gerit. Caules graciles, foliosi.

Folia plus minusve congesta, petiolata, ovato-lanceolata, acuminata, acuta, membranacea. Racemus multiflorus. Bracteae racemi elongatae, lineari-lanceolatae, scariosae, margine glandulosae. Sepala lateralia irregulariter ovato-lanceolata, obtusa, uninervia, apice carinata. Sepalum dorsale anguste ellipticum, obtusum, apice incrassatum. Petala anguste elliptica, obtusa, uninervia, apice incrassata. Labellum in calcar conicum productum; lamina labelli suborbicularis. Columna generis.

Terrestrial herb 17.5-21.5 cm. tall, in facies very similar to *V. albida* Bl. Stems slender bearing several foliaceous bracts near the base, leafy above. Leaves crowded, including the petiole up to 10 cm. long, 1.5-3 cm. wide; lamina ovate-lanceolate, acuminate, acute, papyraceous. Peduncle slender, including the raceme up to 12 cm. long. Raceme 4-5.4 cm. long, about 1 cm. in diameter near the base, tapering gradually upward, densely many-flowered. Bracts of the raceme up to 1 cm. long, linear-lanceolate, glandular-pubescent on the margin, mid-nerve prominent. Ovary about 7 mm. long, smooth. Lateral sepals 3.5 mm. long, 2 mm. wide, asymmetrically ovate-lanceolate, obtuse, conspicuously thickened at the carinate apex, 1-nerved. Dorsal sepal 3.5 mm. long, narrowly elliptical, obtuse, with the blunt apex considerably thickened, 1-nerved. Petals adherent to the dorsal sepal, 3.5 mm. long, 1.5 mm. wide, narrowly elliptical, obtuse, thickened at the tip, 1-nerved. Labellum calcarate, 6 mm. long from the tip of the spur to the tip of the expanded lamina; lamina 1.5 mm. long to the opening of the spur, 2 mm. wide, suborbicular, fleshy, distinctly thickened or bicallose at the base in front of the opening to the spur, somewhat thickened at the tip. Spur about 4.5 mm. long, about 1.5 mm. in diameter, tapering gradually to the obtuse or subacute tip; within on the posterior wall hang two pedicellate verruciform appendages, each appendage and its pedicel 1.5 mm. long. Column 2 mm. long.

M a l e k u l a : South West Bay, at sea level, *L. Cheeseman,* s. n. (type in Herb. Kew; duplicate type in Herb. Ames, no. 37831), January 1930.

From *Vrydagzynea elongata* Bl., this species differs conspicuously in having a conical spur with an acute or nearly acute apex. The smaller flowers, dissimilar lip and larger leaves seem to differentiate it from *V. neo-hibernica* Schltr.

Malaxis lunata (Schltr.) Ames in Jour. Arnold Arb. XIII. 129 (1932).

Microstylis lunata Schlechter in Fedde, Rep. Spec. Nov. IX. 162 (1911).

Aneityum: Hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896; near Anelgauhat, *Dr. Morrison,* s. n., June 1896; ascent of peak south of Ithumu, 1000-1835 feet altitude, *Dr. Morrison,* s. n., June 30, 1896.

Oberonia sp.

Aneityum: Gulley near Anelgauhat, *Dr. Morrison,* s. n., June 13, 1896.

This material collected by Dr. Morrison is in fruit and indeterminable. In general appearance the plant resembles *O. Betchei* Schltr. It also suggests *O. ensiformis* Lindl.

Oberonia sp.

Aneityum: Anelgauhat to Anumy, *Dr. Morrison,* s. n., June 25, 1896.

Sterile specimens that are vegetatively similar to *O. aporophylla* Reichb. f.

Oberonia glandulosa Lindley, Fol. Orch. Oberonia, 6 (1859).

Efate: Undine Bay, *Dr. Morrison,* s. n., August 18, 1896. Aneityum: Hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896 (fruiting specimen).—Also Tahiti.

Liparis elegans Lindley in Wall. Cat. no. 1943 (1828), nomen; Lindley, Gen. & Sp. Orch. 30 (1830).

Aneityum: Peak between Anumy and Ithug, *Dr. Morrison,* s. n., June 26, 1896.—Also Malay Peninsula and Borneo.

Chrysoglossum aneityumense Ames, sp. nov.

Rhizoma gracile, verisimiliter statu juvenili vaginis laxis vestitum. Folium chartaceum, anguste ellipticum, subacutum, basi rotundatum in petiolum sulcatum attenuatum. Scapus erectus, pauciflorus. Sepala lateralia oblongo-lanceolata, acuminata, cum labello saccum scrotiformem formantia. Sepalum dorsale simile. Petala lanceolata, acuta. Labellum conspicue trilobatum; lobi laterales anguste semielliptici, valde obtusi; lobus medius semiorbicularis; discus inaequaliter tricarinatus. Columna crassa, apice alata, basi in pedem producta.

Rhizome slender, partly concealed by the fibrous remains of sheathing bracts, 2-4 mm. thick in dried specimens. Leaf including the petiole 7.5-9.5 cm. long, about 3.5 cm. wide, chartaceous when dry, narrowly elliptical, subacute, rounded at the base; petiole about 1.5 cm. long, terminal on a slender abbreviated pseudobulbous stem. Scape, the continuation of a leafless stem arising from near the base of the leaf, including the few-flowered raceme about 1 dm. long. Raceme 4-6 cm. long, loosely five- to eight-flowered, flowers about 1 cm. apart. Bracts

of the inflorescence about 1 cm. long, lanceolate, acute, membranaceous. Pedicels slender, including the ovary 1.5 cm. long, sharply curved. Lateral sepals 1 cm. long, 4 mm. wide, oblong-lanceolate, acuminate, acute, forming with the labellum a conspicuous scrotiform sac, spreading. Sac 5 mm. long, about 3 mm. in diameter. Dorsal sepal about 1 cm. long, 3.5 mm. wide, similar to the laterals. Petals 9 mm. long, about 3 mm. wide, lanceolate, acute, spreading. Labellum connected with the sac by a narrow claw, including the sac 1.4 cm. long, conspicuously 3-lobed; lateral lobes 4 mm. long, 2 mm. wide, narrowly semi-elliptic, rounded at the apex, separated from the middle lobe by an abbreviated isthmus; middle lobe semi-orbicular, 6 mm. long, 8 mm. wide; disc 3-carinate with the outer carinae auriculate near the base of the lateral lobes and extending nearly to the center of the middle lobe where they become conspicuously elevated into semi-elliptical plates, the central carina is shorter than the lateral ones and hardly expanded at the tip. Column fleshy, free portion 4.5 mm. long, produced at base into a conspicuous foot, at the summit becoming conspicuously winged round the androclinium.

Aneityum: Anelgauhat, below 800 feet altitude, *Dr. R. Morrison,* s. n., June 1896 (type in Herb. Kew; duplicate type in Herb. Ames, no. 39055).

The closest ally of this species appears to be *Chrysoglossum papuanum* (Schltr.) J. J. Smith which differs from the New Hebridean plant in having the lateral lobes of the labellum acute and in having slightly different carinae on the disc. Vegetatively these species are very similar with flowers that are about equal in size.

Dendrobium calcaratum A. Richard, Sert. Astrol. 18. t. 7 (1834).

Aneityum: Hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896; between Anelgauhat and Anumy, *Dr. Morrison,* s. n., June 25, 1896; ascent of peak south of Ithumu, at 1000-1835 feet altitude, *Dr. Morrison,* s. n., June 30, 1896.—Already found on Vanikoro in the Santa Cruz Islands.

Dendrobium gnomus Ames, sp. nov.

Caules dense caespitosi, graciles, perbreves. Folia disticha, linearioblonga, apice inaequaliter bidentata. Racemi pauciflori, laterales. Sepala lateralia mentum obtusum formantia, triangularia, acuta, valde membranacea. Sepalum dorsale lanceolatum, acutum. Petala lanceolata. Labellum elongatum, simplex, infra medium constrictum, bicallosum, supra medium in laminam lanceolatam acutam expansum. Columna generis.

Roots finely fibrous, white, smooth. Stems up to 3.5 cm. long, about

2 mm. in diameter when dry, yellow, deeply furrowed longitudinally, four- to eight-jointed, with the internodes about 5 mm. long and nearly equal in diameter. Leaves alternate, 1.7-2.1 cm. long, 2.5 mm. wide, linear-oblong, tapering gradually toward each end, unequally bidentate at the blunt apex. Leaf-sheaths somewhat complanate, heavily nerved, remaining as loose fibres at the nodes of the stem after the fall of the leaves. Inflorescence breaking forth at the nodes of the naked stems; rachis about 6 mm. long. Bracts of the inflorescence scarious, those subtending the flowers lanceolate, acute. Flowers several, bright purple, about 11 mm. long from the tip of the dorsal sepal to the tip of the mentum formed by the lateral sepals, membranaceous. Lateral sepals slightly spreading, triangular, acute, including the mentum 1 cm. long, about 3 mm. wide near the middle; mentum 4 mm. long, about 2.5 mm. in diameter, closed in front, protuberant anteriorly at the tip. Dorsal sepal lanceolate, acute, 5.5 mm. long, 2 mm. wide. Petals lanceolate, acute, erect, diverging slightly from the dorsal sepal, 5 mm. long, about 1 mm. wide. Labellum 8 mm. long, expanded above the middle into an elliptic-lanceolate lamina which is 2 mm. wide, basal portion narrowly oblong, slightly constricted near the base of the expanded upper portion with a rounded thickening or callus on each side of the constriction. Column produced into an elongated foot.

Santa Cruz Group: Vanikoro, in rain forest at 800 meters altitude, growing in moss, *S. F. Kajewski,* no. 605 (type in Herb. Ames, no. 38083), November 11, 1928 (flowers bright purple, very pretty; (only one specimen seen).

One of the smallest representatives of the Pedilonum section of *Dendrobium,* about equal in size to *D. cyanocentrum* Schltr.

Dendrobium Mohlianum Reichenbach f. in Bot. Zeit. xx. 214 (1862).

Dendrobium neo-ebudanum Schlechter in Bull. Herb. Boiss. ser. 2, vi. 456 (1906).

Aneityum: Between Anumy and Ithug, at 2300 feet altitude, *Dr. Morrison,* s. n., June 26, 1896; hills near Anumy, *Dr. Morrison,* s. n., June 6, 1896. Eromanga: Peak south of Dillon's Bay, *Dr. Morrison,* s. n., August 5, 1896; Traitor's Head, summit of old crater growing on rotten wood, 2400 feet altitude, *L. Cheeseman,* no. 72, August 21, 1930 (flower pale vermillion, not deep).—Also Fiji Islands and Samoa.

Dendrobium Mooreanum Lindley in Jour. Hort. Soc. vi. 272 in footnote (1851).

Dendrobium Fairfaxii Rolfe in Gard. Chron. ser. 3, v. 798 (1889).

Aneityum: Peak south of Ithumu, *Dr. Morrison,* s. n., June

30, 1896 (flower generally pure white, labellum greenish, veined with purple); Anelgauhat to Anumy, *Dr. Morrison,* s. n., June 25, 1896; peak between Anumy and Ithug, *Dr. Morrison,* s. n., June 26, 1896. Efate: Undine Bay, *Dr. Morrison,* s. n., August 27, 1896. Eromanga: Hill south of Dillon's Bay, *Dr. Morrison,* s. n., August 5, 1896.

Dendrobium occultum Ames, sp. nov.

Caules lageniformes vel cylindracei, prope apicem diphylli. Folia oblongo-lanceolata, apiculata, in sicco chartacea. Flores singuli. Sepala lateralia mentum elongatum formantia, oblonga, prope apicem attenuata, apiculata. Sepalum dorsale elliptico-oblongum, apiculatum. Petala oblanceolata, apice rotundata, breviter apiculata. Labellum oblanceolatum, apice apiculatum, prope basim lamella transversa ornatum; discus leviter tricarinatus. Columna generis, clinandrio denticulato.

Stems 1.5-2.5 cm. long, lageniform or cylindrical, bearing at the summit two obliquely ascending leaves, when young concealed by tubular sheaths which persist as stiff erect fibres at the nodes on the mature stems. Leaves 8-13 cm. long, up to 1.5 cm. wide, oblong-lanceolate, apiculate, papyraceous when dry. Flowers 2.8 cm. long, borne singly at the nodes of the leafless stems. Pedicel with the ovary 8 mm. long, ascending. Lateral sepals including the mentum 2.8 cm. long, about 5 mm. wide, oblong, gradually tapering from above the middle to an acute apiculate tip, the nerves somewhat raised on the outer surface in dried specimens, the central one subcarinate near the apex; mentum 8.5 mm. long, slender, 4 mm. in diameter, tapering gradually to the tip where it is about 1 mm. in diameter, closed in front for about one-half of its length. Dorsal sepal 2 cm. long, 5 mm. wide, elliptic-oblong, tapering to an apiculate tip, narrowed toward the base, mid-nerve lightly carinate toward the distal end. Petals about 1.9 cm. long, 7 mm. wide across the upper third, about 2 mm. wide near the base, oblanceolate, rounded at the apex, shortly apiculate, 5-nerved. Labellum about 2 cm. long, about one-third shorter than the lateral sepals, oblanceolate, apiculate, about 6 mm. wide above the middle, the three central nerves more or less prominent, the middle one subcarinate toward the base of the disc; 6 mm. from the base there is a fleshy transverse callus. Column 9 mm. long; clinandrium denticulate.

Santa Cruz Group: Vanikoro, in the moss on rain-forest trees, at 800 meters altitude, *S. F. Kajewski,* no. 604 (type in Herb. Ames, no. 38084), November 11, 1928 (flowers, some cream with yellow-edged labellum, others are purple-pink, but all have the yellow-edged labellum).

Apparently a close ally of *D. asperifolium* J. J. Smith, but the leaves and flowers are not verrucose. In habit similar to *D. pentapterum* Schltr. and *D. Cuthbertsonii* F. v. Muell. The specific name alludes to the tendency of the flowers to be hidden by the moss in which the plants were growing.

Dendrobium purpureum Roxburgh, Fl. Ind. III. 484 (1832).

Dendrobium Morrisonii Schlechter in Bull. Herb. Boiss. ser. 2, VI. 456 (1906).

Aneityum: Hill northeast of Anelgauhat, *Dr. Morrison,* s. n., June 18, 1896; near Anumy, *Dr. Morrison,* s. n., June 1896; hills between Ithumu and Anelgauhat, *Dr. Morrison,* s. n., July 6, 1896 (flowers white).—Also Moluccas.

Dendrobium Quaifei Rolfe, *ined.*

Dendrobium Qnaifei Guillaumin in Bull. Soc. Bot. France, LXIV. 707 (1927), *sphalm.*

New Hebrides: Santo Peak, 4500 feet altitude, *W. T. Quaife,* s. n., May 1903.

The narrowly oblanceolate petals and the dissimilar stelidia of the column separate this species from *D. pseudo-Tokai* Kraenzl. Apparently it is closely allied to *D. montis-yulei* Kraenzl., but it differs sufficiently in the flowers to be regarded as distinct. In habit it is very similar to *D. Mooreanum* Lindl., but from that species it differs conspicuously in having much smaller flowers and a different labellum.

The type consists of a single plant: Caules plus minusve 28 cm. longi, verisimiliter caespitosi, usque ad apicem dilatati, 6 mm. in crassitudine, valde sulcati, flavidi, ad apicem triphylli. Folia plus minusve 9 cm. longa, usque ad 3.3 cm. lata, valde coriacea, apice bidentata. Inflorescentia 20 cm. longa; pedunculus infra racemum plus minusve 11 cm. longus, paucibracteatus. Racemus plus minusve sex-florus, 9 cm. longus. Bracteae racemi vix 5 mm. longae, lanceolatae, concaviusculae, acutae. Pedicelli ascendentes, graciles, plus minusve 3 cm. longi. Sepala lateralia 2 cm. longa, 5 mm. lata, triangulari-lanceolata, acuminata, acuta, nervo medio prominenti. Mentum 8 mm. longum, curvatum, obtusum. Sepalum dorsale simile. Petala 2.8 cm. longa, 7 mm. lata, membranacea, anguste oblanceolata, quinquenervia, obtusa. Labellum plus minusve 1.9 cm. longum, prope medium 1 cm. latum, trilobatum, lobis lateralibus rotundatis, lobo medio 9 mm. longo, 5 mm. lato, triangulari-lanceolato, acuto. Discus supra medium glaber, infra medium callo bisulcato ornatus. Columna generis, stelidiis recurvatis, acutis, terminalibus instructa.

Dendrobium ruginosum Ames, sp. nov.

Caules caespitosi, inferne graciles, prope apicem in pseudobulbum

longitudinaliter ruginosum flavidum producti. Folia ad apicem pseudobulbi congesta, coriacea, elliptico-oblonga vel lanceolata, acuta. Inflorescentia plus minusve quinqueflora. Sepala lateralia mentum obtusum formantia, oblonga, usque ad apicem attenuata, acuta, extus carinata. Sepalum dorsale lanceolatum, apiculatum. Petala prope basim valde angustata, membranacea, supra basim suborbicularia, acuta. Labellum trilobatum. Discus glaber, callo elongato truncato ornatus. Columna generis.

Roots glabrous, whitish, coarsely fibrous. Stems caespitose, 25 cm. tall, seven-jointed; the uppermost internode conspicuously swollen and pseudobulbous, yellow, 7 cm. long, up to 1 cm. in diameter when dry, longitudinally sulcate, tapering to a slender base; the remaining internodes slender, 3.7-4.4 cm. long, 2-3 mm. in diameter, smooth, brownish or yellowish, with the fibrous remains of tubular sheaths persisting at the nodes. Leaves terminal on the pseudobulb, coriaceous, three in number, approximate, elliptic-oblong to oblong-lanceolate, tapering gradually toward the tip, 7.5-8.5 cm. long, 1.7-2 cm. wide, obliquely ascending. Inflorescence longer than the leaves, up to 11 cm. long, rigid, slender, about five-flowered, arising from the axil of a leaf or breaking out from the summit of the pseudobulb below the lowermost leaf. Bracts of the inflorescence about 4 mm. long, lanceolate, much shorter than the pedicels, concave or cymbiform, acute. Pedicels slender, with the verruculose ovary 1.7 cm. long, curving sharply toward the upper end. Flowers rather showy with the sepals and petals white and the labellum pale green with purple stripes. Lateral sepals membranaceous, forming a rounded mentum, including the mentum 2 cm. long, about 5 mm. wide across the middle, oblong, tapering from about the middle toward the acute thickened apex, heavily carinate on the exterior surface along the median nerve, the carina extending beyond the apex of the sepal in a sharp apicule. Mentum 5 mm. long. Dorsal sepal 1.7 cm. long, 7 mm. wide across the middle, lanceolate, apiculate, 5-nerved, lightly carinate on the outer surface. Petals membranaceous, 2.4 cm. long, 1.3-1.5 cm. wide, cuneate at the base, dilated upward into a suborbicular or subrhombic lamina, lightly retuse at the apex with an apicule in the sinus or simply acute, 5-nerved at the base. Labellum about 2 cm. long, 3-lobed; lateral lobes rounded, 1.2 cm. long from the rounded tips to the point of insertion at the base of the labellum, about 5 mm. wide; middle lobe about 7 mm. long, 7 mm. wide, apiculate. Disc smooth, with a heavy truncate median keel on the inner surface which extends to the middle of the labellum. Column about 9 mm. long.

Santa Cruz Group: Vanikoro, in rain-forest at 800

meters altitude, *S. F. Kajewski,* no. 606 (type in Herb. Ames, no. 39056), November 11, 1928 (orchid growing on rain-forest trees; petals white, labellum pale yellow with purple stripes; only two specimens seen).

This species is allied to *D. atroviolaceum* Rolfe, but varies from it in having smaller, differently colored flowers and differently formed petals. The three-lobed labellum serves to distinguish it from *D. Mooreanum* Lindl. and the very dissimilar petals differentiate it from *D. Quaifei* Rolfe, its closest ally in the New Hebrides and Santa Cruz Islands.

Glomera Macdonaldii (Schltr.) Ames, comb. nov.

Glossorrhyncha Macdonaldii Schlechter in Fedde, Rep. Spec. Nov. III. 19 (1906).

E r o m a n g a : Traitor's Head, summit of old crater on mossy trunk of Metrosideros, 2400 feet altitude, *L. Cheeseman;* no. 70, August 21, 1930 (straggling semi-erect branches, flower white).—Already reported from Aneityum.

The type was collected by Macdonald on Aneityum. In the Kew Herbarium there is a sterile specimen collected by Dr. Morrison on Aneityum, between Anelgauhat and Anumy on June 25, 1896. Vegetatively this specimen is very similar to the one from Eromanga collected by Cheeseman.

Phajus amboinensis Blume in Mus. Bot. Lugd.-Bat. II. 180 (1856).

A n e i t y u m : *Dr. Morrison,* s. n., June 22, 1896.—Also Java and Amboina.

This is a small flowered form of the species.

Eulophia macrostachya Lindley, Gen. & Sp. Orch. 183 (1833).

A n e i t y u m : Anelgauhat, on ground under trees, *Dr. Morrison,* s. n., June 9, 1896. E r o m a n g a : Terrestrial, *Dr. Morrison,* s. n., July 18, 1896.—Also Ceylon, Java, Sumatra, Dutch New Guinea and Borneo.

The specimens on which my determination is based have somewhat smaller flowers than usual. Vegetatively the specimens cited above approach *E. novo-ebudae* Kraenzlin in Guillaumin in Bull. Soc. Bot. France, sér. 5, v. 301 (1929), but the labellum is very different if Kraenzlin's description of admittedly poor material is dependable.

Phreatia calcarata J. J. Smith in Bull. Dép. Agric. Indes. Néerl. no. 19, p. 31 (1908).

B a n k s I s l a n d s : Vanua Lava, rain-forest, 3120 feet altitude, *L. Cheeseman,* s. n., October 29, 1929 (flowers white, growing on rotten bole).—Also Papua.

Thrixspermum sp.

Eromanga: Dillon's Bay, *Dr. Morrison*, s. n., August 5, 1896.

There are three specimens, all without flowers, although one is said to have had pale yellow flowers. Vegetatively this species resembles *Thrixspermum Vanoverberghii* Ames, a native of the Philippine Islands.

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THE CHROMOSOME COMPLEMENT OF CYPHOMANDRA BETACEA

THOMAS W. WHITAKER

With six text figures

The genus *Cyphomandra* contains thirty or more species of herbs, shrubs, and small trees, all of which are of South American origin (Bailey, 1925). It is technically distinguished from *Solanum* by the fact that the two cells of the anther are separated by a thickened connective, which appears as a ridge on the back of the anther.

Cyphomandra betacea (Cav.) Sendt., the so-called "Tree Tomato," is cultivated, to a certain extent, by the natives of the American tropics for its edible fruit. It was first described in 1801 under the name *Solanum betaceum* Cavanilles. Later it was placed in the genus *Cyphomandra* by Sendtner (1845).

In the course of an investigation of the chromosome number and behavior of various members of the Solanaceae, several plants of *C. betacea* were made available to the writer through the courtesy of Professor Karl Sax. A preliminary examination indicated that the meiotic divisions were irregular, and, for this reason, it was thought that a further cytological examination would contribute some information on the cause and effect of the irregularities noted at meiosis.

Attention was focused on three points: (1) A description of the nature of the irregularities taking place during meiosis; (2) the effect of these irregularities on the amount of pollen sterility; (3) the probable taxonomic relations of *C. betacea* from a study of its cytology.

Observations on the meiotic divisions were made from aceto-carmine smear preparations. Preparations showing microspore divisions are, as a rule, unsatisfactory when prepared by the usual methods, because the pollen grains are thick and opaque. By fixing with aceto-carmine and exerting considerable pressure on the cover slip, combined with gentle heating of the slide, excellent preparations were secured.

C. betacea has twelve pairs of chromosomes. The haploid complement is shown particularly well at metaphase of the microspore division (Fig. 1). It is apparent that the attachment constrictions are approximately median in all cases, and there are no conspicuous morphological variations among the individuals of the haploid set. In root tip preparations, twenty-four chromosomes are plainly discernible. The somatic chromosomes are characteristically long and slender. Each

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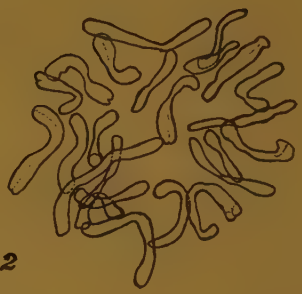

2

FIGURE 1. THE 12 GAMETIC CHROMOSOMES IN THE FIRST DIVISION OF THE MICROSPORE. — FIGURE 2. THE 24 SOMATIC CHROMOSOMES AT METAPHASE. × 2100.

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4

5

6

FIGURE 3. THE 12 BIVALENT CHROMOSOMES AT METAPHASE IN THE FIRST DIVISION OF THE POLLEN MOTHER CELL. — FIGURE 4. FIRST METAPHASE SHOWING ONE UNIVALENT AT EACH POLE, THE REMAINDER AT THE EQUATOR. — FIGURE 5. SHOWS THE SAME SITUATION AS IN FIGURE 4 BUT WITH TWO BIVALENTS SHOWING PRECOCIOUS DIVISION. — FIGURE 6. SHOWS A LAGGING PAIR OF CHROMOSOMES. × 2100.

chromosome has a median attachment constriction, and several have secondary constrictions (Fig. 2).

Good figures of diakinesis were difficult to obtain, owing perhaps to the unusual amount of chromatin contained in the relatively small nucleus, but where satisfactory figures were examined, it is clear that two of the bivalents have only one terminal chiasma. The remaining bivalents have at least two chiasmata and, in certain cases, as many as three chiasmata.

The structure of the individual chromosomes is very clear at metaphase of the first meiotic division, and the spiral chromonemata are easily visible. At this point several abnormalities were observed to interrupt the ordinary sequence of the meiotic process. Briefly these abnormalities were: (1) One and sometimes two of the univalents were located at each pole, while the remainder of the set were still at the equator (Fig. 4); (2) two pairs of chromosomes frequently showed precocious division (Fig. 5); (3) lagging of one pair of chromosomes was repeatedly observed (Fig. 6). It is estimated that these abnormalities occurred in about 50% of the cases.

The irregularities observed at meiosis are reflected in the pollen, since fully 25% of the pollen grains are aborted. The percentage of aborted pollen in some flowers is much greater, reaching an extreme limit of 50% pollen sterility.

DISCUSSION

On comparing the size of the metaphase chromosomes of *C. betacea* with those of *Solanum Capsicastrum* (arbitrarily selecting *S. Capsicastrum* as a representative of the other Solanaceae), it was found that the chromosomes of *C. betacea* averaged slightly over twice as long as those of *S. Capsicastrum* in similar preparations at the same stage. This same relationship existed regarding the width of the chromosomes. The diameters of the pollen mother cells of *C. betacea* and *S. Capsicastrum* stand in the ratio of 1:1.33. Thus the volume of the pollen mother cells of *C. betacea* is approximately three times that of *S. Capsicastrum.*

Vilmorin and Simonet (1928) have reported the chromosome number of a considerable range of solanaceous plants. In their studies they have found an arborescent species of *Solanum* (*S. glaucum*) whose chromosome complement consists of twelve large, irregularly shaped chromosomes. This is the only species of the Solanaceae yet reported where the individual chromosomes approach in size those of *C. betacea.*

Aside from the striking difference in the size of the chromosomes and pollen mother cells, *C. betacea* is very similar, cytologically, to other

members of the Solanaceae. The closely related genera *Salpichroa, Solanum,* and *Lycopersicum* all have a basic number of twelve chromosomes and ring bivalents. In this connection it is interesting to note that although the individual chromosomes of *C. betacea* are considerably larger, both in width and in length than those of other species of Solanaceae, the type of association is identical with that found in species with smaller chromosomes.

The amount of pollen sterility (25%) found in *C. betacea* is quite unusual in a pure species, although by no means exceptional. Professor Jack, of the Arnold Arboretum, informs me that *C. betacea* is considered a good species and that it is quite unlikely that it could have hybridized with any other member of the genus, as it is very distinct from other species of *Cyphomandra.* Therefore the irregularities at meiosis and high pollen sterility cannot be accounted for by assuming that the plants under consideration were a direct result of hybridization with a closely related species.

It has been suggested that the irregularities under consideration might be due to the fact that the plants were grown under a lower temperature than that to which they are accustomed in their native habitat. Acting on this suggestion, the writer has examined pollen of plants from Cuba. (This pollen was supplied through the kindly cooperation of Mr. R. M. Grey of the Atkins Institution of the Arnold Arboretum.) Pollen sterility of the Cuban plants averages approximately 5%, compared with an average sterility of 25% of plants flowering at the Bussey Institution. It appears as if this considerable difference in pollen sterility is significant, particularly since the pollen sterility was as high as 50% in a few cases of the plants grown under different environmental conditions.

There is good evidence that meiosis in plants is, in some measure, disturbed by lowering of the temperature. Belling (1925) first suggested that pairing of the chromosomes might be more easily disturbed by cold in tropical or subtropical plants than would be the case with plants of temperate regions. He has also suggested that cold treatment might be an important means of inducing tetraploidy in tropical plants. Sax (1931) kept plants of *Rhoeo discolor* at about 45 degrees Fahrenheit for several days. The pollen mother cells of these plants showed a complete lack of pairing of the chromosomes at the first meiotic division. When the cold treated plants were again placed under normal conditions, the ordinary meiotic behavior was restored.

Since the plants of *C. betacea* were not critically tested in a similar manner to find out if the temperature was the factor responsible for the

pollen sterility observed, it is impossible to state with certainty that temperature was the environmental variable.

However, what little evidence available is highly suggestive and indicates that the irregularities during meiosis are directly traceable to the low temperatures prevailing when the plants were examined. The manner in which temperature seems to operate, in this case, is by reducing the number of chiasmata in certain chromosomes, which results in very loose pairing at diakinesis.

This naturally leads to speculation in regard to the way in which plants cope with conditions of decreasing temperature. From the evidence at hand, it seems as though the answer to this question may be that decreasing temperature causes greater variability, which, in turn, increases the chances of a mutant's being produced that would be better able to survive under the new set of conditions.

SUMMARY

1. *Cyphomandra betacea* has twelve pairs of chromosomes. The chromosomes of this species are considerably larger than those of any solanaceous plant yet reported.

2. The irregularities which occur during meiosis are discussed, and evidence is brought forth to indicate that these irregularities were due to the temperature conditions prevailing at the time the plants were in flower.

3. Pollen sterility averaging about 25% has been found in the plants examined. This value, when compared with 5% pollen sterility found in plants grown in Cuba, is considered significant and may be attributed to the difference in temperature under which the plants were grown.

4. The indications are that temperature operates on the meiotic division by reducing the number of chiasmata per bivalent in certain chromosomes.

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LITERATURE CITED

Bailey, L. H. (1925). Manual of cultivated plants. pp. 851. (Macmillan Co. New York.)

Belling, John (1925). Production of triploid and tetraploid plants. (Jour. Hered. **16**:463–464.)

Sax, Karl (1931). Chromosome ring formation in Rhoeo discolor. (Cytologia **3**:36–53.)

Sendtner, O. (1845). Monographia Cyphomandrae, novi Solanacearum generis. (Flora **28**:161–176.)

Vilmorin, Roger de & Marc Simmonet (1928). Le nombre des chromosomes chez les Solanées. (Verh. V. Internat. Kongr. Vererb. Wiss. Berlin, 1520–1536.)

STUDIES ON THE PRECIPITIN REACTION IN PLANTS
III. A BIOCHEMICAL ANALYSIS OF THE "NORMAL PRECIPITIN REACTION"[1]

KENNETH S. CHESTER AND THOMAS W. WHITAKER

With four text figures

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[1]For parts I and II see Vol. XIII. 52-80 and 285-296.

I. INTRODUCTION

Thirty years ago Marshall Ward concluded his researches on the parasitism of fungi (22) with the words: "Immunity depends entirely upon the physiological reactions of the protoplasm of the fungus and the cells of the host. In other words, infection and resistance to infection depend on the power of the fungus protoplasm to overcome the resistance of the cells of the host by means of enzymes or toxins, and reciprocally on that of the protoplasm of the cells of the host to form anti-bodies which destroy such enzymes or toxins." This statement, at the time little more than a shrewd conjecture, inaugurated an epoch of new conceptions in phytopathology and physiology. Soon there appeared the classic studies of Bernard in which it was shown that the symbiosis between the orchid and its mycorrhizal partner is not the static, passive equilibrium which it had heretofore been considered, but that it is an active, dynamic parasitism in which the orchid host controls through the mechanism of specific internal prophylaxis the erstwhile parasitic invader, that it is in the words of Gäumann "a parasitism with the brakes on" ("ein abgebremster Parasitismus") (9).

Long before this time, however, the conception of a dynamic internal prophylaxis had served to inspire fruitful researches in human medicine. Ward's analogy was but an echo of the doctrine of acquired immunity in animals. It was thus necessarily resultant that the unexperienced phyto-immunologist should look for guidance to his more experienced colleagues in the field of human immunology. Phyto-immunology has assuredly profited from this new application of medical experience, but there is a grave danger in attempting to transplant *in toto* the techniques and conclusions of one branch of science to the very different soil of a kindred science. There is an unavoidable tendency to interpret *analogies* as *homologies*, failing, however, to consider the inherent differences which characterize the materials of the two sciences.

As early as 1902 (11, 16) phenomena in plants resembling immunological behavior in animals had been observed. Plant saps were found to act toward animal proteins in a fashion resembling that of immune blood in the presence of its specific antigen; they agglutinated and hemolyzed blood corpuscles and precipitated foreign proteins. In succeeding years the literature has become replete with similar observations and in almost all cases the terminology and interpretations of human medicine have been bodily appropriated in the establishment of the science of plant immunity. As the most striking and pertinent example of such non-critical appropriation of zoöimmunitary concep-

tions must be cited the researches dealing with the reactions of grafted plants. The work of Kostoff (12) has already been discussed in this series of studies. It is sufficient to state that this worker found in studying the *Solanaceae* that aqueous extracts of certain plants precipitated *in vitro* in the presence of other such extracts, that after grafting two such plants together the intensity of the precipitation sometimes increased, that in certain cases the overlayering of two extracts resulted in a clear zone at the plane of contact, that the plastids of grafted plants sometimes showed a clumping together in masses, and that various malformations and abnormalities accompanied these other phenomena. All of this, without reserve, was interpreted in the terms of antibody formation: the precipitating action was due to "precipitins" (which term has a very specific meaning in zoöimmunity); the clear zones resulted from the activity of "lysins"; the clumping of the plastids was an "agglutination." Kostoff's work has been accepted as a most conclusive link in the chain of proof that plants may elaborate antibodies of the zoöimmunitary type (2, 8). Indeed one worker, Silberschmidt (21), has published a long and painstaking critique of Kostoff's work suggesting numerous modifications based entirely upon the conception that the reactions observed are of the nature of immunological reactions in animals, although Silberschmidt recognizes in the display of reactions certain differences from the reactions of animal immunology.

It is manifest that if the reactions observed in the *Solanaceae* are *homologous* with the zoöimmunological reactions then a most important step has been taken in the demonstration of antibody formation in plants. If, on the other hand, the reactions observed in the *Solanaceae* are only *analogous* to those in serology, if they are not characterized by the same properties and are not of the same significance as are the reactions in animal immunology, then it is of paramount importance to point out their true significance, be it immunological or merely biochemical in its more limited sense. For this reason the present study was undertaken, in order to determine the nature of the reactions heretofore described as immunological. A short preliminary account of this analysis has already been published (5), and it is the purpose of the present study to substantiate the preliminary account with experimental data.

II. MATERIALS AND METHODS

The plants employed in this study were of species from a variety of herbaceous and woody families; they are enumerated below:

LEGUMINOSAE: *Robinia fertilis* Ashe.

OLEACEAE: *Ligustrum ibota* Sieb. & Zucc., *L. obtusifolium* Sieb. & Zucc., *L. vulgare* L.; *Syringa vulgaris* L.

PLATANACEAE: *Platanus acerifolia* Willd.

ROSACEAE: *Prunus Armeniaca* L. var. *ansu* Maxim. and var. *"Mikado"* Hort.

SAXIFRAGACEAE: *Ribes Carricrei* Schneid. (*R. glutinosum albidum* Jancz. × *R. nigrum* L.); *Hydrangea paniculata* Sieb. var. *grandiflora* Sieb.

SOLANACEAE: *Atropa Belladonna* L.; *Browallia viscosa* HBK.; *Capsicum frutescens* L.; *Cyphomandra betacea* Sendt.; *Datura ferox* L., *D. innoxia* Mill., *D. metel* L., *D. Wrightii* Hort. = *D. meteloides* DC.); *Lycopersicum cerasiforme* Dun.; *Nicotiana alata* Lk. & Otto, *N. acuminata* Grah., *N. Cavanillesii* Dun., *N. glauca* Grah., *N. glutinosa* L., *N. Langsdorffii* Weinm., *N. nudicaulis* Hort., *N. Palmeri* Gray, *N. paniculata* L., *N. plumbaginifolia* Viv., *N. Rusbyi* Britt., *N. rustica* L., *N. Sanderae* Hort. Sand. (*N. alata* × *Forgetiana*), *N. suaveolens* Lehm., *N. sylvestris* Speg. & Comes, *N. Tabacum* L., *N. tomentosa* R. & P., *N. trigonophylla* Dun.; *Petunia violacea* Lindl.; *Physalis peruviana* L.; *Salpiglossis sinuata* Ruiz & Pav.; *Solanum Capsicastrum* Link, *S. Melongena* L., *S. nigrum* L., *S. tuberosum* L.

The Solanaceae enumerated were greenhouse plants for the use of which the writers are indebted to Professor E. M. East. The woody plants were mature specimens in the ornamental collection of the Arnold Arboretum. Leaves from the woody species were collected on Sept. 2, 1931, dried at room temperature for one month, and then pulverized and stored in wide-mouthed glass bottles until used. The solanaceous leaves were collected as needed. In nearly all cases reported below the solanaceous extracts were made from leaves which had been dried in a dessicating oven at 55°C. for from one to three days and then pulverized.

The **method of extraction** varied according to the requirements of the various experiments. It is necessary to distinguish here between pre-extraction and solution. The procedure of pre-extraction and its significance in protein reactions has been thoroughly discussed by Silberschmidt (21). Briefly the purpose of a pre-extraction process is to remove from the tissues substances, chiefly lipoids, which give non-protein reactions and hence tend to render the readings inaccurate. Pre-extraction usually involves extraction with strong alcohol, ether, and chloroform. Such pre-extraction was employed in certain of these experiments to determine its effect upon the resulting reactions (see pages 000 and 000), but was not used in the remainder of the experiments because it was the purpose of these experiments to determine the reactive constituents of extracts of the type used by Kostoff, in which no pre-extraction was employed.

The leaf powders or pulps were then extracted in the solvent designed to dissolve the reactive principles. In most of the experiments reported below distilled water was used as a solvent. Physiological NaCl solution was used in some cases in place of the water, particularly in certain of the dialysis experiments in which it was desirable to avoid the autoprecipitation of extracts due to the withdrawal of electrolytes. Here, too, in certain experiments to determine the relative value of various solvents, a selection of several processes was employed, but, unless otherwise stated in the experiments to follow, the reader may understand that distilled water was used as a solvent. The concentration customarily employed was one part by weight of fresh leaf pulp to two parts of solvent or one part dried leaf powder to ten parts of solvent. This concentration is here referred to as normal concentration (N) and further dilution is indicated by the appropriate fraction (N/2, etc.). Extraction of reactive substances occupied two hours at room temperature or 18 hrs. at 2°C. in all cases, it having been previously ascertained that there is no significant difference in the reactivity of extracts prepared under such conditions.

The **clarification** of the extracts was accomplished by various means depending upon the difficulty in obtaining clear solutions. The dried leaves yielded clear extracts much more readily than fresh leaves, and on the whole little difficulty was experienced in obtaining crystal-clear extracts. The customary procedure was to extract in an open beaker or large test-tube, stirring from time to time, to pour the pulp and extract into a funnel lined with a fine filter paper (C. S. & S. #589) which in turn was lined with sterile gauze. The gauze was then drawn together to form a bag, the liquid squeezed out into the filter paper, and the clear filtrate used in testing. In a few cases it was necessary to repeat filtration through one or two other papers. When dealing with a very small quantity of tissue (we have been able successfully to extract .1 gm. of tissue in 1 cc. of water) it was customary to centrifuge, rather than filter, drawing the clear supernatant liquid off into another tube with a fine pipette. In only a few cases was it impossible to obtain clear extracts. *Salpiglossis*, *Browallia*, and *Hydrangea* gave the most trouble in this connection, and when it was necessary to use extracts from such species the procedure employed was to catch the filtrate immediately it came through the filter and to test it at once. In any case these extracts were used in very few of the experiments reported below. *Nicotiana Rusbyi* proved to be rather refractory in aqueous solution but where physiological saline was used as a solvent, the extracts were satisfactory. On standing for several days, even at

2°C., there was frequently an autoprecipitation of the extracts, but in no case of the many tested did the subsequently cleared extracts exhibit an altered reactivity. Extracts which were to be preserved for any appreciable amount of time were covered with ¼"-½" of toluol and kept in the cold. Bacterial or fungus contamination rarely occurred and then only in extracts which had stood for a long period in the cold. Although it was ascertained that such contamination did not affect the reactivity of the extracts (see page 000) such extracts were not used in the tests reported below unless specifically so stated.

For testing, the procedure described in an earlier paper (4) was employed. With a fine pipette .2 cc. of the liquid of greater density was introduced into the usual small test tubes and over this was layered an equal quantity of the second liquid to be tested. The length of time of reading varied with the requirements of the experiments from 5 to 40 minutes. Where gross results were desired a duration of 5-10 minutes was satisfactory. In fact the reactions observed were usually so marked that a maximum reading could be made in a very few minutes, while a continuation of the time merely permitted the precipitates to diffuse through the extracts making readings less accurate. In a few of the experiments where only gross differences were of importance, the tests were performed by mixing equal quantities of the two extracts in 3" test tubes and observing the resulting cloudiness in comparison with the unmixed liquids. Throughout this paper, in any case, each experiment is a unit, performed under homogeneous conditions and for the purpose of demonstrating one point. The whole series of experiments were not devised for numerical comparison with each other, and accordingly the demands of scientific caution have been met by performing each experiment under its own set of standard conditions. The numerical value assigned to any given reading was according to one of two scales. The first scale, and that employed in nearly all cases, is that described and figured in an earlier paper of this series (4) in which a trace of a reaction (t) is succeeded by reactions of strength 1, 2, 3, and 4 (maximum ever observed). A more accurate measure of reactivity which was employed in a number of cases was to calculate the numerical sum of the readings (according to the first scale) at 1, 5, 10, 20, 30, and 40 minutes respectively. To be sure neither scale is wholly free from error, and both are arbitrary measures; but it is the belief of the writers that the readings within the limits of these experiments are accurate within the range of 1 unit on the t-1-2-3-4 scale and that a greater accuracy of reading is not compatible within the inherent variability of the material and technique. All of

the readings reported in this paper were made by the same observer, a second observer being consulted in case of doubt.

Numerous **controls** were employed in each experiment. The extracts were tested against the pure solvents and against such other fluids as the individual experiments required.

Protein determinations by the use of the Millon and xanthoproteic tests were resorted to, in general, only in the dialysis experiments and then only for the purpose of obtaining a measure of the efficiency of the dialyzing membranes. Silberschmidt (21) feels that such determinations should be made on all extracts. When it is possible to say with assurance that one is dealing with protein reactions of the zoöimmunitary type, then the protein tests will be of value, but since the aim of the present study was to determine the nature, protein or otherwise, of the reactive substances, it was not felt necessary to employ protein tests in all cases. Similarly other tests (for chloride, oxalate, calcium, etc.) were employed only as the specific experiments required them.

Since a brief summary of most of these results has already been published (5) it is possible to state at the outset the sequence of points which are to be considered in the present paper. It was pointed out in this earlier summary that a reaction responsible for the majority of positive tests which have been observed is the reaction between calcium and oxalate ions in two respective extracts. It then being possible to eliminate from discussion the calcium oxalate reaction, attention may be focussed on the remaining reactions. Having ascertained that there are at least three other such reactions, designated here respectively as the AB, MN, and XY reactions, and their respective properties having been determined, it is finally possible to analyze the experimental data of Kostoff, Silberschmidt, East and Chester with respect to the rôle of these various reactions in the published accounts of the "normal precipitin reaction," and to point out the significance, immunological or otherwise, of the precipitin reaction in plants. Hence the remainder of this paper will concern itself with answering the following questions: 1. What is the nature of the reactions occurring between *Platanus, Robinia, Ribes,* and *Prunus?* 2. What is the evidence supporting the statement that a calcium oxalate reaction is responsible for the majority of the recorded positive precipitations? 3. What proof is there as to the presence and number of other reactions in the plants studied? 4. What are the properties of these additional reactions? 5. What light does this study cast upon the previously recorded studies of the precipitin reaction in plants? 6. What is the theoretical and practical significance of the precipitin reaction in plants?

III. THE NATURE OF THE REACTIONS OCCURRING BETWEEN PRUNUS, PLATANUS, ROBINIA, AND RIBES

Early in this study the question was considered whether or not the precipitations observed were due to inorganic constituents. Attention was diverted from the theory that they might be explainable in such simple terms because of the following facts: (a) the specificity exhibited by the reaction (4) appeared hardly in conformity with such a simple explanation; (b) accepting Kostoff's work at face value it would be inconceivable from the complexity of his results and their apparent immunological significance that he was dealing with such a simple reaction; (c) when in one preliminary experiment extracts of *Platanus* and *Prunus* were ignited at red heat and then the ash redissolved in water, the reaction had disappeared. The influence of the immunological school of thought was so strong that the writers as well as their various associates were strongly inclined to believe the reactions highly complex in nature. The series of experiments devised accordingly emphasized the proof of the presence or absence of protein in the reactive fractions of the extracts. With the elimination of protein as a reactive principle, attention was directed to the other complex chemical constituents of the extracts, and not until most of the major chemical groups had been eliminated was attention forcibly drawn to the function of the inorganic constituents. The evidence is accordingly of two sorts, negative evidence that the proteins and various other chemical groups could not be responsible for the reactions, and positive evidence that calcium-oxalate was responsible. The present section will treat with the first of these subjects, i. e. the negative evidence, while the following section will deal with the proof of the calcium oxalate reaction.

The first experiments performed were with the species of woody plants enumerated above, but particularly with *Prunus Armeniaca "Mikado," Platanus acerifolia, Ribes Carrierei,* and *Robinia fertilis.* Later the results were extended to include the other species listed above. Of the extracts employed the *Prunus* tests strongly against *Platanus, Robinia* and *Ribes,* while the latter three are negative to each other.

A. Effect of Salt Concentration on the Reactions

As a primary step in the investigation of the nature of these reactions it was felt desirable to study the effect of certain physical factors on the reaction, in order to determine the optimum conditions for reaction, to observe the specific effect of variation of such factors, and to obtain a standard technique for subsequent work. The following experiment

was therefore first set up for the purpose of studying the effect of the variation of the electrolytic concentration in the extracts.

Five samples of dry leaf powder of *Platanus*, *Ribes*, and *Robinia* respectively were extracted in the usual manner in ten times the weight of solvent. For solvents were used a series of phosphate buffer solutions prepared according to the system worked out by Cohn (6). All the buffers had the same pH, namely 6.0 (which is relatively close to the normal pH of these extracts). On the other hand the five buffers of each of the three series had salt concentrations respectively of .06M, .12M, .30M, .60M, and 1.2M. This system of buffers has the advantage of introducing only one metallic ion [KH_2PO_4 (acidic) : K_2HPO_4 (basic)]. The fifteen extracts were then cleared and tested against one

TABLE I.

EFFECT ON THE PLATANUS-ROBINIA-PRUNUS PRECIPITIN REACTION OF VARYING THE SALT CONCENTRATION.

		Robinia...Phosphate content (Cohn)						Platanus...Phosphate content (Cohn)					
		.06 M	.12 M	.30 M	.60 M	1.2 M	Total	.06 M	.12 M	.30 M	.60 M	1.2 M	Total
	.06 M	15.0	5.0	5.5	3.0	2.5	31.0	15.0	14.5	9.5	4.0	0.0	43.0
Prunus phosphate content	.12 M	11.0	9.5	10.0	9.5	4.5	44.5	17.0	15.0	8.0	5.5	2.0	47.5
	.30 M	11.0	11.0	6.0	7.0	5.5	40.5	16.0	15.0	7.5	4.0	3.0	45.5
	.60 M	11.0	10.5	9.5	4.0	3.0	38.0	17.0	15.0	9.5	2.0	0.5	44.0
	1.20 M	15.0	9.5	4.5	2.5	2.5	34.0	15.0	12.5	4.5	0.0	0.0	32.0
	Totals	63.0	45.5	35.5	26.0	18.0		80.0	72.0	39.0	15.5	5.5	

another. The results of these tests are given in Table I. The numerical values assigned to the various reactions were computed by summating the strengths of reaction at the various time intervals from 1 to 40 minutes. For control experiments the various extracts of *Robinia* were tested against one another in all possible combinations; the same was done with the extracts of *Prunus* and *Platanus*. All extracts were tested against the various strengths of pure buffer, and finally the *Robinia* series was tested against the *Platanus* series. All of these control tests were completely negative.

Several points are brought out by this table. In the first place one observes that although *Robinia* and *Platanus* behave in the same fashion with regard to salt concentration, both differ in marked fashion from *Prunus*. Variation of salt content through this wide range hardly affects *Prunus* while *Robinia* and *Platanus* show a steady decline in reactivity as the strength of salt increases. This fact would indicate that the reactive substances in the two types of extract are different, a

situation not in conformity with that in animal immunology. Since the higher concentrations of salts used in this experiment are far greater than occur in ordinary water or saline extracts, this experiment also reveals that with regard to salt concentration the conditions under which the experiments have previously been performed have been optimal. And finally the relative insensitiveness, at least of *Prunus,* to salt concentration is not in accordance with the great dependence of serological reactions upon salt concentration, and affords a first suggestion as to the non-protein nature of the reaction.

B. Effect of Hydrogen ion Concentration on the Reactions

This first experiment having revealed that the reaction is apparently not affected by the weaker salt concentrations used, and having indicated the best salt concentrations for further study, the next step was to vary the pH while holding the salt concentration near the optimum. For this purpose *Robinia* and *Prunus* were chosen. Each was divided into 9 samples and each series of samples was extracted in the usual manner in a series of buffers (Cohn's system) all having the same salt concentration (.06M) but having a variety of hydrogen ion concentrations. The range of pH varied from 5.2 to 8.4, since this is the maximum range possible with Cohn's system. However, it must be emphasized that the extreme values here used (5.2 and 8.4) with this buffer system are susceptible to a somewhat greater error than the remaining values. This pH range extends from a point somewhat more acid than the ordinary pH of the extracts (except *Prunus* which may fall as low as 4.0-4.5) to a point far more alkaline than has been observed in any normal extract. The extracts having been prepared, all the possible combinations were made in precipitin tubes and as in the preceding experiment the readings were made at intervals for 40 minutes and then summated. The results of this experiment are shown in Table II.

The results of this experiment are of interest in confirming the earlier findings of Kostoff that the reaction bears no relation to pH of the extracts. It is seen from a study of the table that no significant difference is to be observed throughout the whole of the pH range studied. This does not directly afford evidence as to the nature of the reaction, since the precipitin reaction in animals is also independent of pH within reasonable limits (17) but it does indicate that there is no necessity for a careful checking of the pH of every experimental series.

As controls for this experiment each member of both series was tested against the complete series of pure buffers, the reactions in all cases being negative.

A rather interesting phenomenon was observed in connection with this experiment, namely that variation of pH in such a graded series revealed that the plant extracts thus prepared contain coloring substances with the behavior of chemical indicators. In both *Prunus* and *Robinia* the pH series showed a progressive darkening of color as one passed from the acid to the alkaline extremes. In *Prunus* the color ranged from pale lemon yellow at 5.2 to molasses brown at 8.4. The *Robinia* extracts were all lighter than those of *Prunus* and ranged from

TABLE II.

EFFECT ON THE PRUNUS-ROBINIA PRECIPITIN REACTION OF VARYING THE pH OF THE SOLVENT

		Robinia...pH									
		5.2	5.6	6.0	6.4	6.8	7.2	7.6	8.0	8.4	Total
Prunus pH	5.2	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0	126.0
	5.6	17.0	17.0	17.0	17.0	17.0	17.0	15.0	12.5	12.5	142.0
	6.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	15.0	15.0	149.0
	6.4	17.0	17.0	17.0	17.0	17.0	14.0	14.0	14.0	14.0	137.0
	6.8	17.0	17.0	17.0	16.0	16.0	16.0	15.0	15.0	15.0	140.0
	7.2	17.0	17.0	17.0	17.0	17.0	15.0	14.0	14.0	14.0	138.0
	7.6	17.0	17.0	17.0	17.0	17.0	17.0	16.0	16.0	16.0	150.0
	8.0	17.0	17.0	17.0	17.0	15.0	14.0	13.0	13.0	13.0	143.0
	8.4	14.0	14.0	14.0	14.0	14.0	14.0	13.0	13.0	13.0	124.0
	Total	147.	147.	147.	146.	144.	138.	131.	127.	127.	

very pale lemon at 5.2 to amber at 8.4. Differences in the color of the extracts were also observed in the preceding experiment, where the extracts in more concentrated phosphate were slightly darker, but the effect was much less than in the present experiment. The variation of color intensity with pH has been repeatedly observed in other subsequent experiments. It is of value since by comparing two extracts which have been placed under different experimental conditions (as in enzyme digestion) it is possible to make a rough estimate from the color as to the pH effect. This fact was made use of in later experiments. While speaking of the color of the extracts it might be well to mention, in passing, the effect of dialysis on the pigment in these extracts. In the many dialyses which have been employed in this study it has always been observed that the completely dialyzed dialyzates

always retained the same dark color as the undialyzed controls, i. e. the pigment is non-dialyzable. However a colorless color-base does pass readily through the membranes and if this colorless diffusate is boiled a rich brown color results, presumably from its oxidation.

Beside the color change due to pH there is another pH effect on the extracts which should be mentioned, namely, the tendency for more alkaline extracts to become cloudy or opalescent. Silberschmidt recognized this fact (l. c. p. 146: "das Filtrat wurde leicht trüb, vermutlich, da es etwas alkalisch war"). This effect was not noted below the point of neutrality, but it has been repeatedly observed in alkaline extracts. It is easily eliminated by making the extracts neutral or slightly acid, which results in a re-solution of the solid suspension.

A second experiment on the effect of pH is incorporated into an experiment on heat in the following section (page 130). The result of this experiment was to demonstrate that the reaction does not take place at pH 1.4-1.5 or at pH 8.8. This failure in reactivity may be due to one of two possibilities: either (a) the reactive substances are present in extracts which have been so titrated but are unable to react because of the excessive alkalinity or acidity of the solutions, or (b) the process of titration has resulted in a precipitation of the active ingredients, the reactivity of which is irrevocably lost if the coagulum is removed. The answer as to which of these two possibilities is the correct explanation is seen in the following experiment.

TABLE III.

EFFECT OF REMOVAL OF ALKALINE COAGULUM ON PRECIPITIN REACTION IN PRUNUS-PLATANUS-ROBINIA.

		Platanus		Robinia	
		Normal:	Alkaline supernatant:	Normal:	Alkaline supernatant:
Prunus	Normal:	4	4	3	3
	Alkaline supernatant:	4	4	3	3

Normal extracts of *Prunus*, *Platanus*, and *Robinia* were prepared (in distilled water). Each was divided into two fractions, one fraction being titrated to pH 9.0 with KOH. The abundant precipitate was then centrifuged away, and the supernatant fluid titrated with HCl back to approximately the normal pH. The extracts were then tested as is shown in Table III.

A study of this table reveals that the alkaline coagulum does not contain the reactive substances but that the supernatant fluid from

such coagulation does contain the reactive principles in undiluted strength, and that, by comparison with Table V, the reaction in the latter table is inhibited not by absence of reactive substances but by an excessively high pH, the reaction being fully restored when the pH is again brought to normal.

C. Effect of Heating on the Reactions

Having now determined the effects of salt concentration and pH upon the reactions, the next important step was to determine the effect of heat upon the extracts, for a sharp decline in reactivity of extracts which have been heated to temperatures of 60°-80°C. would yield strong evidence as to the nature of the reaction. Several experiments in heating the extracts have been performed, all yielding comparable results. As a preliminary word it should be mentioned that serologically active protein is usually coagulated at about 60°C., sera heated above this point and subsequently cleared having lost their specific reactive power. However, a somewhat different display of behavior might be anticipated with the plant extracts since according to Osborne (19, p. 65) "the seed proteins differ in a marked degree from the animal proteins, for most of them are very incompletely coagulated by heating their solutions even to boiling, and many of them are not coagulated at all under these conditions." Neutral and alkaline protein solutions are heat-coagulable only with difficulty or not at all, so that in the experiments reported below the extracts were all prepared under such conditions as to give them a slightly acid reaction.

In the first heat experiment performed, extracts of *Robinia* and *Prunus* were employed. A quantity of normal extract was prepared for each of the species, the solvent used being a Cohn's phosphate buffer of pH 6.0 and salt concentration of .12M (6). The two extracts were then each divided into 10 samples. One sample of each was untreated, the other 9 samples were each heated in water baths for ½ hour at varying temperatures from 30°C. to 100°C., so as to afford a complete series for *Robinia* and for *Prunus*. All the extracts were then clarified by centrifuging where necessary, and tested in all possible combinations. Clarification was unnecessary throughout in *Prunus* as the very slight opalescence which developed at the highest temperatures was so slight as not to interfere with reading the reactions. On the other hand, the *Robinia* extracts threw out a very dense and flocculent precipitate which began at 60° and became intense at 70°-100°C. The removal of these precipitates, however, as will be seen below, had little effect on the reaction. As controls for this experiment the tests of the

treated extracts against the normal extracts and then against one another suffice to eliminate possibilities of error due to non-experimental variables. The results of this first experiment are given in Table IV.

TABLE IV.

EFFECT OF HEAT UPON THE PRUNUS-ROBINIA PRECIPITIN REACTION.

		Robinia.....Heated 1/2 hour at temperature:									
		None	30°	40°	50°	60°	70°	80°	90°	100°	Total
	None	17.0	17.0	17.0	17.0	17.0	12.0	11.0	10.0	7.0	125.
	30°	17.0	17.0	17.0	17.0	17.0	12.0	11.0	10.0	7.0	125.
Prunus...	40°	17.0	17.0	17.0	17.0	17.0	12.0	11.0	10.0	7.0	125.
Heated 1/2 hour at:	50°	17.0	17.0	17.0	17.0	17.0	12.0	11.0	10.0	7.0	125.
	60°	12.0	12.0	12.0	11.0	10.0	7.5	6.5	5.5	6.5	83.
	70°	12.0	12.0	12.0	9.0	8.0	7.5	5.5	5.5	6.5	78.
	80°	11.0	12.0	10.0	8.0	8.0	7.5	5.5	5.5	5.5	73.
	90°	10.0	10.0	10.0	7.0	6.0	5.5	5.0	5.5	3.0	62.
	100°	9.0	9.0	9.0	7.0	6.0	5.5	4.0	5.5	3.0	58.
	Total	122.	121.	121.	110.	106.	81.5	70.5	67.5	52.5	

As will be seen from a study of the data here presented there is a decline in the reactivity of both extracts on being heated above 60°C. At first glance this would seem to indicate the direct effect of a protein coagulation. Indeed, such was the conclusion of the writers on first viewing the results. But subsequent experiments have shown this conclusion to be erroneous. The decrease observed at 60° is, to be sure, probably an effect of the heat coagulation, but an indirect effect, the coagulum of protein having by physical action withdrawn from solution, in coagulation, some of the other, non-protein substances present in the extracts.

As a further test of the effect of heating it was resolved to heat the extracts more thoroughly in an endeavor completely to eliminate the reaction. Accordingly normal extracts were prepared of *Prunus* and *Robinia* in distilled water. Each extract was divided into three samples, one sample being reserved as a control, the other two being titrated with HCl to pH 1.4 and with NH_4OH to pH 8.8 respectively. The unaltered extract of *Prunus* had a pH of 4.3, that of *Robinia* 4.8. Each sample was again divided into two, one fraction being heated, the other

being retained as a control. Heating was accomplished by autoclaving with flowing steam at a pressure of 2 lbs. for three hours. The extracts were then cleared by centrifuging and tested as in Table V.

In all cases there were coagulations due to heating as well as coagulations due to acidification and alkalinization. These were more con-

TABLE V.

EFFECT OF LONG CONTINUED HEATING ON THE PRUNUS-ROBINIA PRECIPITIN REACTION.

		Prunus....Unheated:			Prunus.....Heated:		
		pH 1.4	pH 4.3	pH 8.8	pH 1.4	pH 4.3	pH 8.8
Robinia (Unheated)	pH 1.5	0			0		
	pH 4.8		17			17	
	pH 8.8			0			0
Robinia (Heated)	pH 1.5	0			0		
	pH 4.8		17			17	
	pH 8.8			0			0

spicuous in *Robinia* than in *Prunus*. In view of this fact, the results of Table V are most interesting. There was no perceptible influence on the reaction due to this prolonged heating. All the reactions were repeated in parallel several times and viewed by two observers to confirm this point. It is thus manifest that the coagulum due to heating does not contain the reactive substances in these extracts and that accordingly the reaction is either due to a non-protein or to a protein highly resistant to heat. The effect of pH is here also worthy of comment. In the extracts which had been titrated there was a complete loss of reactivity even in the unheated controls. The statements made in the preceding section must accordingly be amended to include the conception that alteration of pH to very high or very low values completely eliminates the reaction, which view is compatible with the other findings reported. It need hardly be emphasized, however, that such pH values as 1.4 and 8.8 never have been observed to occur in extracts prepared in the ordinary fashion.

The effect of pH on the color of the extracts was again noted in this experiment, that of both extracts varying from light amber at 1.4 to dark mahogany at 8.8.

As further controls for this experiment the *Robinia* extracts at vari-

ous pH values, both heated and unheated were tested *inter se* with completely negative results. The same was true of the *Prunus* extracts. This completely eliminated any artefact due to the experimental procedure. Furthermore, reactions between any two extracts of different pH were not included because of the artefact reaction introduced through pH coagulation.

D. Effect of Dilution on the Reactions

One of the most striking effects of the precipitin reaction in animal serology is the zone effect, whereby an excess of reactive substance may inhibit the reactive display, where there is an optimum concentration of reactive principles, which optimum if passed in either direction, results in a decrease of reactivity. The question arises whether such a zone phenomenon is characteristic of the reactions being studied in this paper. The answer to this question will at once afford a significant

TABLE VI.

EFFECT OF EXTRACT DILUTION ON THE PRUNUS-PLATANUS PRECIPITIN REACTION.

		Platanus extract...Normality:													
		10	5	2	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Total
Prunus extract... Normality	10	29.0	27.0	24.0	20.0	15.0	14.0	12.0	10.0	7.0	4.0	1.5	1.0	0.0	164.5
	5	27.0	25.0	20.0	17.0	16.0	12.0	11.0	9.0	5.5	1.0	0.0	0.0	0.0	143.5
	2	24.0	21.0	16.0	15.0	9.0	8.0	7.0	5.5	4.0	0.0	0.0	0.0	0.0	109.5
	1	18.0	17.0	15.0	14.0	8.5	6.0	5.5	3.5	3.0	0.0	0.0	0.0	0.0	90.5
	1/2	15.0	12.0	12.0	11.0	8.0	6.0	4.5	3.0	2.5	0.0	0.0	0.0	0.0	73.0
	1/4	12.0	12.0	11.0	9.0	7.0	5.5	3.5	3.0	2.5	0.0	0.0	0.0	0.0	65.5
	1/8	12.0	12.0	11.0	7.0	5.0	4.5	2.5	0.0	0.0	0.0	0.0	0.0	0.0	54.0
	1/16	11.0	11.0	10.0	5.5	5.0	4.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	49.0
	1/32	7.0	6.0	4.0	3.0	1.5	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	23.0
	1/64	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0
	1/128	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1/256	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1/512	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Total:	156.	145.	128.	101.	75.0	61.5	48.5	34.0	24.5	5.0	1.5	1.0	0.0	

comparison between the plant precipitations and those of serology and at the same time instruct as to the desirability of controlling this possible variable in subsequent work. Accordingly the following experiment was devised.

Normal extracts of *Platanus* and *Prunus* were prepared as usual.

Each extract was then boiled down to 1/10 its volume (reference to the work in heat effects assuring one that this would not seriously interfere with the reactivity of the extracts). They were then centrifuged. Using these 10 normal supernatants as bases, progressive series of dilutions were made. Then each grade of *Prunus* was tested against the various grades of *Platanus* according to the scheme of Table VI.

The readings were made by the method of summation defined above, and the totals plotted against the normalities of the extracts as in Figure I.

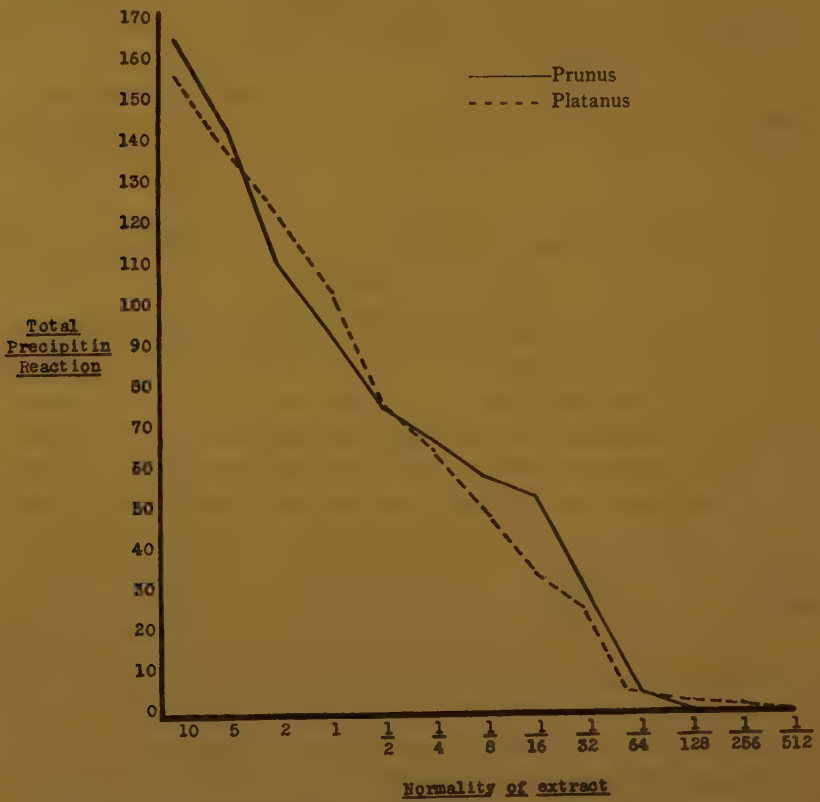


FIGURE I. VARIATION IN THE PRUNUS-PLATANUS PRECIPITIN REACTION WITH PROGRESSIVE DILUTION.

It is at once apparent from a study of Table VI and Figure 1 that dilution of the extracts causes only a regular dilution of reactivity. There is not the slightest indication of a zone effect. In this respect, then, the plant reaction under consideration exhibits a marked differ-

ence from precipitin reactions in animals, and the evidence against an homologous reaction with that of mammalian blood is accordingly strengthened. The relative regularity of the curves also affords a measure of the relative accuracy of the method of reading, since there is no appreciable deviation at any point of the curves.

E. Effect of Dialysis on the Reactions

The next, and probably the most important step in determining the nature of the reactions under consideration is the dialysis of the extracts with subsequent testing. If the reactive substances are completely or nearly completely held back by protein-impermeable dialyzing membranes, then there is strong but not absolute proof that the reaction is due to proteins or to radicals bound to proteins. If, on the other hand, the reactive substances easily pass through membranes which are independently proved to be impermeable to proteins, then the evidence is conclusive that the reactive substances are non-protein in nature. Accordingly great stress is here laid on the dialysis experiments reported.

The technique of dialysis varied at the start until a technique was found which proved to be wholly satisfactory. For membranes, collodion and cellophane were first employed but they proved to have numerous disadvantages in comparison to the parchment which succeeded them. The collodion membranes, in addition to the labor of making them, proved much more difficult to handle and there was the greater danger of their rupturing or being imperfectly constructed, while the cellophane (commercial: Dupont Corp. #300, not waterproof) proved to be so frequently imperfect that not more than one out of five sheets was satisfactory. Moreover the cellophane when wet is very easily broken and is handled only with difficulty. A high grade of parchment, first in the form of diffusion cells, later in sheets, proved entirely satisfactory. Although every sheet was tested in these experiments, very few were found to require rejection.

As regards apparatus, the first designed was in the form of the customary shallow tray with a periodic water change, in which the liquid to be dialyzed was placed in diffusion shells. This was efficient but very slow in contrast to the other apparatus described below. Using the diffusion shells two weeks were necessary for a satisfactory dialysis (at 2°C.), and since the time was so extended there was danger of errors due to this long standing.

A dialysis device employed by Dr. F. D. Hagar at the Massachusetts Antitoxin and Vaccine Laboratory was studied and then modified to meet the conditions of this study. The apparatus is constructed according to the plan of Figure II.

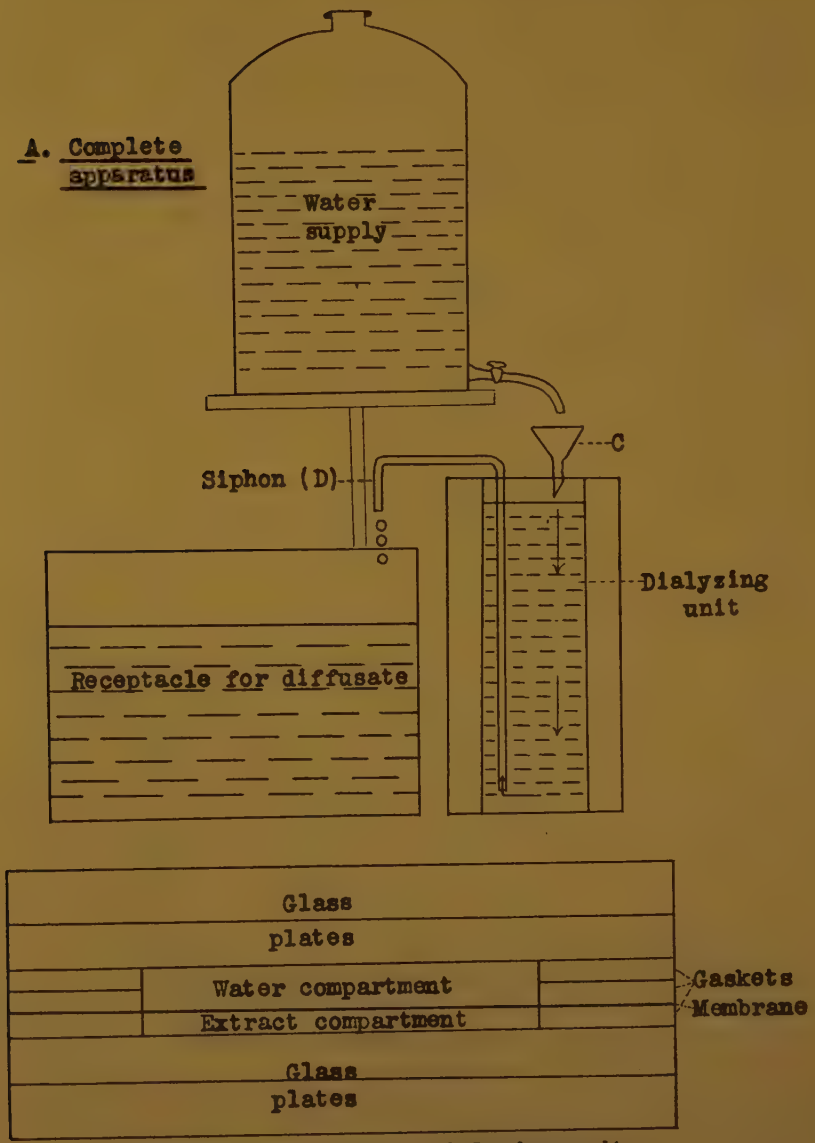


FIGURE II. APPARATUS FOR DIALYSIS OF EXTRACTS.

The dialyzing membrane, cut to a size of 8″ x 4″ (or preferably narrower) is fixed between two U-shaped rubber gaskets of the same size, one gasket being twice the thickness of the other. An automobile inner tube of heavy, smooth rubber may be used for the construction of the gaskets. These gaskets are in turn clamped between two heavy glass plates. The glass must be rather thick (¾″, or we have used two plates on each side of ⅜″-½″ thickness each). On the side of the membrane separated from the glass by the thinner gasket is placed the extract to be dialyzed. The other side of the membrane is in contact with a moving stream of water, entering at the funnel C and leaving by the siphon D. Such an apparatus affords a high degree of efficiency and has the advantages of affording a continuous flow, yet of being small enough to be placed in an ordinary sized refrigerator. About 25 cc. of extract may be dialyzed at a time, although by constructing the apparatus on a smaller scale this could be reduced to 5 cc. if desirable. The water flow is at about the rate of two litres per day, and dialysis for 1-2 days has given perfectly satisfactory results. At first the dialyses were carried out at 2°C., but later tests showed that a dialysis for 24-48 hours at room temperature did not seriously interfere with the reactivity or clearness of the liquids, and accordingly most of the experiments reported below were performed at room temperature.

Using such an apparatus the diffusate may be saved and tested in addition to the dialyzate if such proves desirable. The experiments on the *Prunus-Platanus-Ribes-Robinia* reactions soon showed that the reactive principles passed rapidly through the membranes, and accordingly the diffusates were studied. These were evaporated by boiling in small fractions and thus reduced to the original volume of the dialyzed extracts. Control tests were employed in which whole extracts were diluted to 3-4 litres and then boiled down. Since there was no reduction in reactivity in such controls, it was assumed that the technique of boiling down the diffusate was justified.

The efficiency of the membranes was tested in several ways. In the first place, a set of preliminary tests were made with the parchment to determine their permeability. These experiments are tabulated in Table VII. The tests for the presence or absence of substances in dialyzate and diffusate included the Millon and xanthoproteic tests for protein, the Ninhydrin test for protein cleavage products, the iodine test for starch, the Fehling test for sugar, and the silver nitrate test for chloride. These tests are all highly sensitive. The Millon test reveals the presence of one part of peptone in 3000 parts of water, the Ninhydrin test one part of peptone in 50,000 parts of water (water-clear),

and the Fehling test is sensitive to at least one part of glucose in 2000 parts of water (these sensitivities being independently determined for this study). The sensitivity of the $AgNO_3$ test is also very high, that of iodine less so.

TABLE VII.

EXPERIMENTS IN DIALYSIS: TESTING OF PARCHMENT MEMBRANES.

Exp.	Substance dialyzed:	Nature of extract:	Dialysis tests for: Protein: Millon	Protein: Xanthoproteic	Protein: Ninhydrin	Starch: Iodine	Sugar: Fehling	Cl: $AgNO_3$
A	Peptone + glucose	Normal	5		5		5	
"	"	Dialysate	5		5		5	
"	"	Diffusate	0		1		3	
B	Ovalbumin + starch + glucose + NaCl.	Normal	4	4		3	5	5
"	"	Dialysate	4	4		3	4	4
"	"	Diffusate	0	0		1	3	3
C	"	Normal	4	4		3	5	5
"	"	Dialysate	4	4		3	4	4
"	"	Diffusate	0	0		1	3	3

In Experiment A the solution to be dialyzed contained peptone (saturation) and glucose (.2%) in water. Dialysis lasted for 18 hours and there was no continuous flow of water in the apparatus, which contained 30 cc. of dialyzate and 40 cc. of diffusate. At the end of this experiment the tests showed that the peptone had passed through only in very small amount, while the glucose had passed through freely.

In Experiment B the solution to be dialyzed consisted of powdered egg albumen (.5%), soluble starch (.2%), d-glucose (.25%), and NaCl (about .2%). The clear filtered solution was dialyzed for 24 hours with no flow in the apparatus. The tests at the end of this time showed that no albumen was demonstrable in the diffusate, while it was unaltered in strength in the dialyzate, that the sugar and salt had passed through freely, and that starch had passed through but not so freely as the sugar and salt.

Experiment C was a repetition in all respects of Experiment B, and it will be seen that the results were exactly the same as in the preceding experiment.

Thus these three membranes, chosen at random, showed a porosity of the exact type required for this work, and offered confidence that the use of such membranes would be of value in the succeeding experiments.

Beside these preliminary tests, each dialysis by itself was tested for degree of dialysis by employing on controls, dialyzates, and diffusates tests for protein, carbohydrates, and salts. The Millon, Fehling, $AgNO_3$,

and oxalate tests were extensively used. The xanthoproteic and iodine tests were also used but less frequently, because the former is of little value in strongly colored extracts, while the latter is less sensitive than the other tests. It is to be emphasized that there is no intention that the presence or absence of Millon-testable protein directly indicates the presence or absence of (hypothetical) precipitin-active protein. Rather these chemical tests are to be considered tests of the degree of dialysis and nothing more. Thus if the diffusate of any given experiment shows no Millon protein, then it is a natural assumption that all protein is being reasonably well retained, while if the diffusate gives a strong $AgNO_3$ or Fehling test, this is indicative that all crystalloids of the same approximate molecular size are passing freely through the membrane.

In each experiment a control tube of the same extract as was being dialyzed was placed under corresponding conditions, at room temperature or at 2°C. respectively throughout the experiment, and then used as "Normal" in the dialysis tests. The protein, salt, and precipitin tests were all performed in the customary manner with the usual controls. All the dialyses lasted from 1-2 days, and all were performed using distilled water as the fluid outside the membrane. The results of the dialyses with *Prunus, Platanus,* and *Robinia* are here introduced in tabular form (Tables VIII-XI) since space does not permit the introduction of an intimate discussion of each experiment.

Considering first the reactions of *Platanus acerifolia* (Table VIII) the control extracts in every case contained strong protein, chloride, and sugar as is shown by the tests. The controls were all highly and equally reactive with *Prunus* and completely negative with *Robinia,* in conformity with the earlier experiments. In dialysis, except in one experiment (Exp. 9) the Millon protein was completely held back by the membranes, while the salt passed freely through. It is thus highly significant that the diffusates in every case offered strong reactions with the *Prunus,* while the dialyzates, although not negative in but one case, exhibited only very weak reactions. The membrane in Experiment 7 appeared to have been far more retentive than any of the others, as is shown by all the tests, and accordingly the results in this experiment are of less significance than the others. Thus it is evident that there is a definite and strong correlation between the diffusable substances through protein-impermeable membranes and the precipitin potency. The fractionation is not perfect, but in such experiments as these the significance depends not upon a single test but upon the trend exhibited by all. That the positive reactions of the diffusates were not artefacts

TABLE VIII.

EXPERIMENTS IN DIALYSIS: DIALYSES OF PLATANUS.

Nature of extract:	Exp. #:	Dialysis tests for: Protein	Chloride	Sugar	Precipitin tests against normal: Extract:	Test:	Extract:	Test:
Normal	5	4	4	4	Prunus	4	Robinia	0
"	6	4	4	4	"	4	"	0
"	7	4	4	4	"	4	"	0
"	8	4	4	4	"	4	"	0
"	9	4	4	4	"	4	"	0
"	10	4	4	4	"	4	"	0
Dialyzate	5	3	1	0	"	1	"	0
"	6	3	1	0	"	1	"	0
"	7	3	3	0	"	3	"	0
"	8	3	1	0	"	1	"	0
"	9	4	1	0	"	0	"	0
"	10	4	2	1	"	1	"	0
Diffusate	5	0	4	4	"	4	"	0
"	6	0	4	4	"	4	"	0
"	7	0	4	4	"	4	"	0
"	8	0	4	4	"	4	"	0
"	9	1	4	4	"	4	"	0
"	10	0	4	4	"	4	"	0

TABLE IX.

EXPERIMENTS IN DIALYSIS: DIALYSES OF ROBINIA.

Nature of Extract:	Exp. #:	Dialysis tests: Protein	Chloride	Precipitin tests against normal: Extract:	Test:	Extract:	Test:
Normal	19	4	4	Prunus	4	Platanus	0
"	21	4	4	"	4	"	0
"	22	4	4	"	4	"	0
"	23	4	4	"	4	"	0
"	24	4	4	"	4	"	0
"	25	4	4	"	4	"	0
"	26	4	4	"	4	"	0
Dialyzate	19	3	1	"	1	"	0
"	21	4	2	"	2	"	0
"	22	2	1	"	0	"	0
"	23	4	1	"	1	"	0
"	24	3	1	"	1	"	0
"	25	4	2	"	2	"	0
"	26	4	2	"	2	"	0
Diffusate	19	2	4	"	4	"	0
"	21	1	4	"	3	"	0
"	22	2	4	"	3	"	0
"	23	2	4	"	4	"	0
"	24	2	4	"	4	"	0
"	25	1	4	"	3	"	0

TABLE X.

EXPERIMENTS IN DIALYSIS: DIALYSES OF PRUNUS.

Nature of extract:	Exp. #:	Dialysis tests: Protein	Chloride	Sugar	Precipitin tests against normal: Extract:	Test:	Extract:	Test:
Normal	1	4	4	4	Platanus	4	Robinia	4
"	2	4	4	4	"	4	"	4
"	3	4	4	4	"	4	"	4
"	4	4	4	4	"	4	"	4
"	11	4	4	4	"	4	"	?
"	12	4	4	4	"	4	"	?
"	13	3	4	4	"	4	"	2
"	15	4	4	4	"	4	"	4
Dialysate	1	3	1	0	"	0	"	0
"	2	3	1	1	"	0	"	0
"	3	3	1	0	"	0	"	0
"	4	3	1	0	"	0	"	0
"	11	4	2	0	"	0	"	?
"	12	4	2	0	"	0	"	?
"	13	2	1	0	"	0	"	0
"	15	3	2	0	"	0	"	0
Diffusate	1	0	4	?	"	4	"	4
"	2	0	4	4	"	4	"	4
"	13	1	3	3	"	2	"	2
"	15	2	4	4	"	3	"	3

TABLE XI.

EXPERIMENTS IN DIALYSIS: TESTS OF FRACTION AGAINST FRACTION (PRUNUS-PLATANUS)

Prunus: Exp. #	Nature of extract:	Dialysis tests: Protein	Chloride	Sugar	Platanus: Exp. #	Nature of extract:	Dialysis tests: Protein	Chloride	Sugar	Precipitin reaction
11	Dialysate	4	2	0	9	Dialysate	4	1	0	0
12	"	4	2	0	9	"	4	1	0	0
15	"	3	2	0	9	"	4	1	0	0
1	Diffusate	0	4	4	5	"	3	1	0	0
1	"	0	4	4	6	"	3	1	0	0
1	"	0	4	4	7	"	3	3	0	0
1	"	0	4	4	8	"	3	1	0	0
15	"	2	4	4	9	"	4	1	0	0
1	Dialysate	3	1	0	10	Diffusate	0	4	4	0
2	"	3	1	1	10	"	0	4	4	0
3	"	3	1	0	10	"	0	4	4	0
4	"	3	1	0	10	"	0	4	4	0
11	"	4	2	0	9	"	1	4	4	0
12	"	4	2	0	9	"	1	4	4	0
1	Diffusate	0	4	?	10	"	0	4	4	4
2	"	0	4	4	10	"	0	4	4	4
1	"	0	4	4	5	"	0	4	4	4
1	"	0	4	4	6	"	0	4	4	4
1	"	0	4	4	7	"	0	4	4	4
1	"	0	4	4	8	"	0	4	4	4

is demonstrated by the fact that they were absent when tested against *Robinia* and also when tested against one another (additional control tests not shown in the table).

In Table IX the results with *Robinia fertilis* are entirely comparable. Here the retention of protein is not as complete as in *Platanus* (though it must be remembered that the Millon test is positive not only to formed protein but also to certain of its cleavage products, e.g. peptone), but the dialysis results in a fractionation in which one fraction, the diffusate, contains the bulk of the crystalloids, and the other fraction, the dialyzate, the bulk of the protein. The fraction containing most of the crystalloids contains most of the precipitin-reactive substances, and the fraction containing most of the protein shows only weak precipitin reactions.

The results with *Prunus Armeniaca* (Table X) are most striking of all. Here the fractionation resulted in diffusates which were almost or entirely protein-free according to the Millon test but which contained the great bulk of the crystalloids, while the dialyzates showed little protein loss and little crystalloid retention. Subsequent tests have shown that the chloride which seems necessarily retained with the protein is probably bound to the protein and is hence non-dialyzable, since the equally delicate oxalate test in later experiments showed such dialyzates to be perfectly calcium-free. The high-protein, low-crystalloid dialyzates were perfectly negative to active extracts of *Platanus* and *Robinia,* while the high-crystalloid, protein-negative diffusates were richly endowed with precipitin potency.

The next table, Table XI, shows the tests of fraction against fraction. These tests were performed to obtain confirmatory data to Tables VIII-X and also to answer a question that arose from a consideration of the data in these preceding tables. It is known that in animal precipitin reactions the presence of a small amount of salt is necessary in order that the reactions take place. In testing a dialyzate against a diffusate or a normal extract one would expect that if the loss of reaction were due to a loss of salt, the second extract would supply sufficient electrolyte to permit the reaction to take place. Such does not occur (see Tables VIII-X as well as XI). On the other hand, if the reaction is due to protein in either extract it would be highly improbable that the testing together of two Millon-negative diffusates could produce a reaction equivalent in strength to the original reaction of the normal extracts. Granting that a trace of protein, non-testable by the Millon test, may have escaped through the membranes, could this give a positive reaction *equal in strength to the controls?* One could hardly

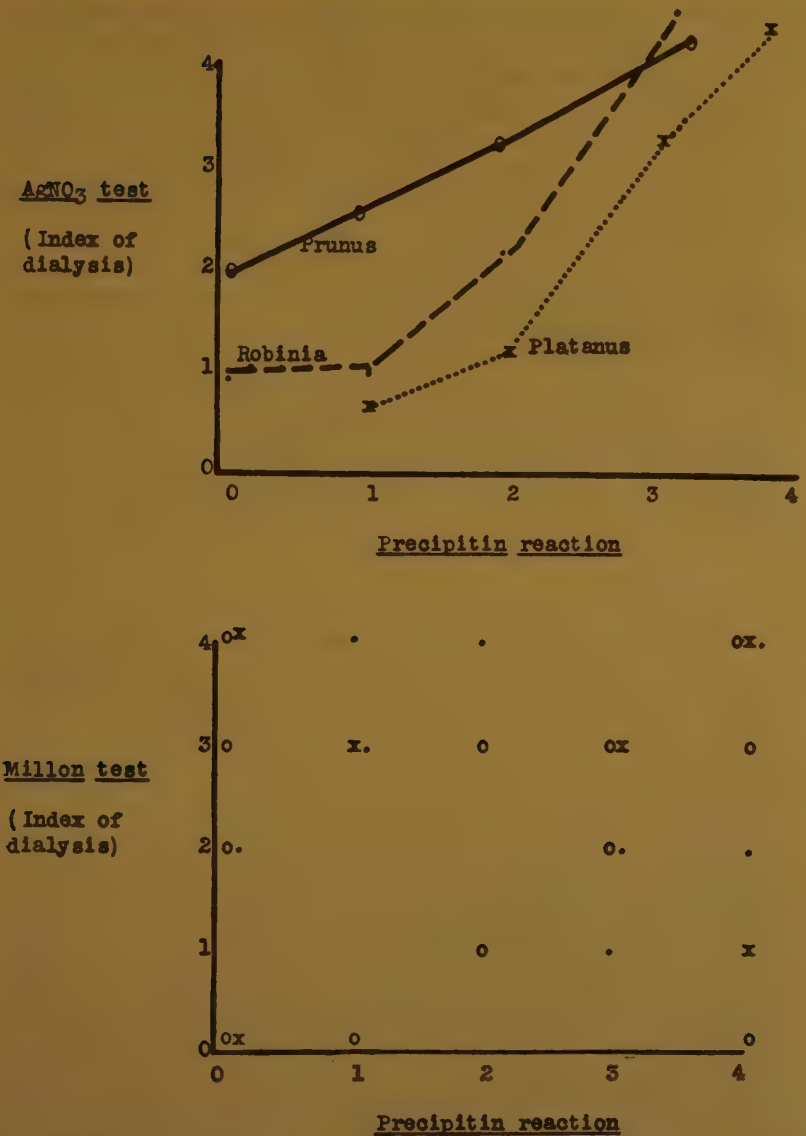


FIGURE III. EFFECT OF DIALYSIS ON THE PRUNUS-ROBINIA-PLATANUS PRECIPITIN REACTION.

KEY: o = Prunus; . = Robinia; x = Platanus. O = no test; 1 = very weak test; 2 = weak test; 3 = moderate test; 4 = strong test.

cling to such a remote possibility in the present case, for the dialyzates are in no case positive to one another nor to the diffusates, while the diffusates are in every case not only positive *inter se,* but positive to a strength *equivalent to that of the normal control reaction.* The results of all these experiments are summarized in the graphs of Figure III which illustrate strikingly that the reactive substances bear a direct and intimate relationship to the dialyzable crystalloids and none whatever to the non-dialyzable proteins.

The experiments in dialysis thus prove definitely that whatever the nature of the *Platanus-Prunus-Robinia* reactions, the substances responsible for them pass freely through membranes highly impermeable to protein. This applied equally to the reactive principles of *Prunus, Platanus,* and *Robinia.* It is thus inconceivable that the precipitin reactions among these species could be due to protein unless one clings to the very doubtful hypothesis that a very small fraction of protein may give as strong a precipitin test as a quantity of protein 10 or 100 times as great, provided that electrolytes be present in sufficient quantity.

F. Effect of Various Solvents on the Reactions

Several types of experiments have been undertaken to demonstrate what solvents will dissolve the reactive principles of *Platanus, Prunus, Robinia,* and *Ribes,* as well as what solvents will denature the reactive principles. The solvents employed have been alcohol, ether, benzol, chloroform, and carbon tetrachloride. The purposes of the experiments were various, first, to investigate the possibility of improving the technique by an extraction process which will eliminate many heterologous substances from the extracts, second, to shed light upon the chemical nature of the active principles by determination of their various solubilities, third, to observe the effect of denaturing, particularly with regard to protein, upon the reaction, and fourth, to determine whether the techniques used by Silberschmidt in pre-extraction and solution exert any significant effect on the reactions at present being considered.

The first experiment performed upon this topic was one designed to determine whether the reactive substances of *Platanus* and *Prunus* are soluble in alcohol of various strengths. Samples of leaf powder of these species were extracted in the customary manner but using as solvents water, 20%, 40%, 60%, 80%, and absolute alcohol. These extracts were filtered, and then tested for their content in protein, sugar, and chloride, and for their precipitin potency. It was not possible to test all the possible combinations since the addition of a strong alcohol to a

water solution or one in weak alcohol resulted in an artefact coagulation effect. The results of this experiment are given in Table XII.

It is seen that there is a steady fall in precipitin potency as the strength of alcohol is increased until there is no reaction in alcohol of 60% or stronger. A comparison with the chemical tests shows that

TABLE XII.

DIRECT EFFECT OF ALCOHOL UPON THE PRUNUS-PLATANUS PRECIPITIN REACTION.

				Prunus: Extracted in alc. of strength:					
				100%	80%	60%	40%	20%	0%
			Protein:	2	2	3	4	1	3
			Sugar:	?	3	3	3	4	4
			Chloride:	0	t	1	2	3	4
	Extr.	Prot.	Sug.	Cl.					
	100%	t	?	0	0.0				
	80%	2	3	t		0.0			
	60%	3	3	1			0.0		
Platanus	40%	4	3	2				11.0	
	20%	4	4	3					13.0
	0%	3	4	4					17.0

there was no correlation between precipitin test and sugar (Fehling) or between precipitin reaction and protein, while there was an excellent correlation between the precipitin reaction and salt. This is of significance only by analogy, but the analogy is striking. It so happened in this experiment that the 20% alcohol solution of *Prunus* showed much weaker protein than either of the adjacent grades, but this is by no means reflected in the precipitin tests. The same reasoning applies to the water extract of *Platanus*. Thus this experiment informs us that the precipitin reactive substances are progressively less soluble in the increasing strengths of alcohol, and that the same is true of the chloride present in the extracts, although it is equally true of neither the protein nor the sugar.

An important question arises from this experiment, namely: Why were the extracts in strong alcohol non-reactive? Was it because the active substances are insoluble in alcohol or was it because they are

denatured by the stronger alcohol? This question was answered by the following experiment.

Leaf powders of *Platanus, Prunus,* and *Robinia* were extracted for 24 hours at 2°C. each with water, 60% alcohol, and 95% alcohol in three lots. The water extracts were then filtered and put on ice. The alcohol extracts were likewise filtered and the filtrates put aside. The alcoholic residues were dried and then extracted in water for 12 hours at 2°C. These last water extracts of the alcoholic residues were then filtered and all the extracts were tested in the manner shown in Table XIII.

TABLE XIII.

EFFECT OF ALCOHOL UPON THE PRUNUS-PLATANUS-ROBINIA PRECIPITIN REACTION.

		Prunus:				
			60% alcohol.		95% alcohol.	
		Water control	Alcohol extract	Water extract	Alcohol extract	Water extract
Platanus	Water cont.	4		4		4
	60% alc. Alc. extr.		1			
	60% alc. Water extr.	4		3		4
	95% alc. Alc. extr.				0	
	95% alc. Water extr.	4		4		4
Robinia	Water cont.	3		3		3
	60% alc. Alc. extr.		1			
	60% alc. Water extr.	3		2		3
	95% alc. Alc. extr.				0	
	95% alc. Water extr.	3		3		3

The results of this experiment are very striking. *Prunus, Platanus,* and *Robinia* all behaved similarly with respect to alcohol extraction. In no case was there evidence that 24 hours in alcohol had to any extent whatever weakened the reaction. Solution in 95% alcohol dissolved none of the reactive principles. Solution in 60% alcohol dissolved a small fraction of the reactive principles in all cases. One may say with assurance, therefore, that the reactive principles involved in these extracts are insoluble but not denatured, under these experimental conditions, by strong alcohol.

Proceeding next to a consideration of the same questions as were

raised regarding alcohol, but respecting lipoid solvents, the following experiment was performed. Dried tissues of *Prunus, Robinia,* and *Platanus* were thoroughly extracted with many changes of (a) ether, (b) chloroform, (c) benzol, and (d) carbon tetrachloride, in separate samples. The pulps were then dried and extracted in water in the customary way. Meanwhile other samples of each species were extracted in water without the lipoid pre-extraction. The extracts were then all tested for their precipitin potency. The scheme followed in testing may be illustrated in a single example (Table XIV).

TABLE XIV.

EFFECT OF LIPOID PRE-EXTRACTION ON PRUNUS-PLATANUS PRECIPITIN REACTION.

	Pre-extraction with ether: Strength of precipitin reaction after:						
Extracts tested together:	1 min.	5 min.	10 min.	20 min.	30 min.	40 min.	Total reac.:
Prunus control + Robinia control..	2	3	3	3	3	3	17
" treated + " control..	2	3	3	3	3	3	17
" control + " treated..	2	3	3	3	3	3	17
" treated + " treated..	2	3	3	3	3	3	17

It will be seen that the ether extraction removed no detectable quantity of the precipitin-reactive substances. Since space is limited the corresponding tables for chloroform, carbon tetrachloride, and benzol are omitted, but were they included they would simply be a duplication of Table XIV. In other words, long continued pre-extraction of these tissues with ether, chloroform, carbon tetrachloride, or benzol in no case resulted in the withdrawal of any appreciable amount of reactive substance. We may thus conclude that the reactive principles here are not of lipoid nature.

Further tests were employed with regard to benzol, in order to confirm the preceding experiment. Leaf powders of *Prunus, Platanus,* and *Robinia* were divided into two samples each. One sample was extracted in water as a control, a second sample was extracted for two hours at room temperature in commercial benzol. The benzol was filtered off, part being saved (A), the remainder being evaporated to dryness and re-dissolved in water (B). The benzol residue was dried and extracted in water (C). The various possible precipitin tests were then performed. The results of the tests showed that in every case the extract prepared from the benzol filtrate was perfectly negative whether the test was performed in benzol (A) or in water (B), while the reactions of the extracts prepared from the benzol residues (C) were equal in strength to those of the control extracts which had experienced no pre-extraction.

A last experiment to test the solubility of the precipitating principles in ether and benzol took the following form. Leaf powders of *Prunus*, *Platanus*, and *Robinia* were divided each into two samples. One sample was extracted directly in water. The other was first extracted in cold C. P. anhydrous ether, the extraction being continued as long as color was present in the filtrate (about 15 changes in 3 hours). These last samples were then divided as follows: 1 gm. of each when dry was placed in a tube with 10 cc. of water and extracted for 2 hrs. at room temperature (Table XV: *Ether controls*). The latter were then fil-

TABLE XV.

EFFECT OF BENZOL PRE-EXTRACTION ON PRUNUS-PLATANUS-ROBINIA PRECIPITIN REACTION.

		Precipitin reac. of:				
		Normal control	Ether control	Benzol filtrate: In benzol	Benzol filtrate: In water	Benz. res. In water
Experimental:	**Experimental:**					
Prunus	+ Platanus	3	3	0	0	3
Prunus	+ Robinia	3	3	0	0	3
Platanus	+ Robinia	0	0	0	0	0
Experimental:	**Normal:**					
Prunus	+ Platanus	3			0	3
Prunus	+ Robinia	3			0	3
Platanus	+ Prunus	3			0	3
Platanus	+ Robinia	0			0	0
Robinia	+ Prunus	3			0	3
Robinia	+ Platanus	0			0	0

tered. Of the ether residue 2.5 gm. of each species was next extracted in commercial benzol. Many changes were employed, extending through two days. The last change was with boiling benzol, the others with cold benzol. After each extraction (lasting about 1 hour), the benzol was decanted and filtered. The filtrate was evaporated in moderate heat. Previous to final evaporation the clear benzol filtrates were tested against one another (Table XV: *Benzol filtrate in benzol*). After complete evaporation the evaporated residue was re-dissolved in water, which was brought to boiling momentarily and then allowed to cool and extract over night. The following morning the water extract was cleared and tested (Table XV: *Benzol filtrate in water*). Meanwhile the benzol residue of powder was dried (with momentary boiling at the start), and extracted in water over night (Table XV: *Benzol residue*). In this, as in all the preceding and subsequent experiments the ratio of dried tissue to solvent was always 1:10, regardless of the solvent or amount of tissue. The results of these tests are seen in Table XV.

These last experiments thus confirm the earlier ones in demonstrating

that thorough extraction with lipoid solvents neither dissolves nor denatures the reactive substances.

As has been mentioned above, Silberschmidt has devoted much of his critique of technique (21) to the methods of pre-extraction and solution. As it was thought that Silberschmidt's results might have depended to considerable extent upon his methods of extraction, and as, further, it was desirable that the results of Kostoff and of Chester be reduceable to the same terms as Silberschmidt's, it was of much interest to determine the effect of Silberschmidt's techniques on our own material. Accordingly the following experiment was designed to include within one experiment all of the techniques described and used by Silberschmidt.

Homogeneous samples of dried leaves of *Prunus*, *Platanus*, and *Ribes* were extracted according to the following scheme:

Experimental tube number:	Pre-extraction:	Solution:
1	None	Water
2	None	Physiological saline
3	None	Water + solid NaCl to form phys. sol.
4	None	Phys. NaCl + MgO to neutrality
5	None	N/20 NaOH
6	95% alc. + 1% tartaric acid (5 hrs.)	Water
7	Ditto	Physiological NaCl
8	Ditto	Phys. NaCl + MgO to neutrality
9	95% alc. + tartaric acid 1% (5 hrs.); ether (5 hrs.); chloroform (5 hrs.); chloroform vapor (18 hrs.).	Water
10	Ditto	Water + solid NaCl to form phys. sol.
11	Ditto	Physiological NaCl
12	Ditto	Phys. NaCl + MgO to neutrality

All of solutions 6, 7, and 8 for any given species were made from the same pre-extracted tissue, and the same applies to 9, 10, 11, and 12 for each species. In pre-extracting there were three changes of each pre-extractant, the first change being momentary, the second lasting ½ hour, the third 1½ hrs. The pre-extracted tissues were then dried and extracted for 2 hours, each in its proper solvent. In decanting, in all the experiments in which this process was employed, it was cus-

tomary to decant repeatedly through the same filter paper, adding the residue in the paper to the leaf tissue being extracted. The various experimental extracts were then tested against normal extracts according to the plan in Table XVI.

TABLE XVI.

APPLICATION OF SILBERSCHMIDT'S METHODS OF PRE-EXTRACTION AND SOLUTION TO THE PRUNUS-PLATANUS-RIBES REACTION.

Extract number regardless of species	Exper. Platan. + Normal Prunus	Exper. Ribes + Normal Prunus	Exper. Prunus + Normal Platan.	Exper. Prunus + Normal Ribes	Exper. Prunus + Ca-free Platan.	Exper. Platan. + Ox-free Prunus	Exper. Ribes + Ox-free Prunus	Exper. Prunus + Ca-free Ribes
1	3	3	3	3	0	0	0	0
2	3	3	3	3	0	0	0	0
3	3	3	3	3	0	0	0	0
4	3	3	3	3	0	0	0	0
5	3	3	3	3	0	0	0	0
6	3	3	3	3	0	0	0	0
7	3	3	3	3	0	0	0	0
8	3	3	3	3	0	0	0	0
9	3	3	3	3	0	0	0	0
10	3	3	3	3	0	0	0	0
11	3	3	3	3	0	0	0	0
12	3	3	3	3	0	0	0	0

In this table "Normal" *Prunus*, *Platanus*, and *Ribes* refer to extracts #1 of these species respectively (aqueous extracts in which there had been no pre-extraction). A word of explanation must be inserted with regard to the last four columns of this table. At the time that this experiment was performed, the authors had already determined that the reaction in these species is due to the interaction of calcium ion in *Platanus* and *Ribes* with oxalate ion in *Prunus*. Therefore it was possible not only to determine the effect of the various extraction methods upon the calcium oxalate reaction, but it was also possible to eliminate the calcium oxalate reaction from consideration and observe whether these various techniques resulted in the demonstration of any additional reaction beyond that due to calcium oxalate. Accordingly, in the last four columns of Table XVI the "Normal" extracts are extracts in which

in the case of *Prunus* the oxalate present had been precipitated by the addition of a slight excess of $Ca(NO_3)_2$, and in the cases of *Platanus* and *Ribes* the calcium present had been precipitated by the addition of a slight excess of $K_2C_2O_4$.

Returning now to the experiment proper, one first observes that none of the various techniques of pre-extraction and solution made the slightest difference in the strength of reaction resulting, in any of the three species used. This demonstrates that there is no step in Silberschmidt's techniques which will eliminate the calcium oxalate reaction, and further that his techniques do not modify the calcium oxalate reaction, in these plants at least. The experiment also further confirms the earlier findings reported in this paper with regard to solubility of reactive substances in the various solvents employed. Lest the objection be raised that there might have been different reactions from the extracts obtained by the different techniques which gave equal (but not homologous) precipitin reactions, one may observe that in the last four columns of this table the elimination of the calcium oxalate reaction completely eliminates all inter-reactivity of the extracts employed. Hence one may conclude that the calcium oxalate reaction is the only one concerned here.

Summarizing, then, the experiments on extraction of the *Prunus*, *Platanus*, *Robinia*, and *Ribes* extracts, one may say that:

1. The reactive substances in these plants are insoluble in strong alcohol, ether, chloroform, carbon tetrachloride, benzol, and 95% alcohol + tartaric acid 1%.
2. They are not denatured by treatment with any of these solvents.
3. They are equally soluble in distilled water and physiological NaCl solution.
4. The reactions take place with equal facility in the presence of NaCl (.85%), MgO, and N/20 NaOH, and in acid and neutral solutions.

G. Effect of Carbohydrate Removal on the Reactions

In order to test the possibility that the reactive principles might be of carbohydrate nature, an experiment was undertaken to eliminate carbohydrates from consideration. The technique of Rimington (20) was employed. Normal extracts of *Platanus*, *Robinia*, and *Prunus* were prepared in the customary manner. To each of these was added a slight excess of neutral lead acetate. There was a voluminous precipitate, containing mucilaginous substances, etc. This precipitate was redissolved by washing and suspending in water and bubbling through H_2S. To the filtrate (neutral $Pb(C_2H_3O_2)_2$ filtrate) was then added about

1/10 the volume of the original extract of NH_4OH solution. A second voluminous precipitate resulted, this containing the bulk of the carbohydrates (alkaline PbAc precipitate). NH_4OH and $Pb(C_2H_3O_2)_2$ were then added in turn until the precipitate was maximum, and the filtrate (alkaline $Pb(C_2H_3O_2)_2$ filtrate) virtually negative to the Molisch test. The alkaline $Pb(C_2H_3O_2)_2$ precipitate was redissolved in acetic acid, and the excess lead was removed from it and from the alkaline $Pb(C_2H_3O_2)_2$ filtrate with H_2S. These solutions were then titrated back to slight acidity and the precipitin tests performed. The H_2S was removed by boiling, although tests showed that it did not interfere with the reaction. The result of these tests is given in Table XVII.

TABLE XVII.

EFFECT OF CARBOHYDRATE REMOVAL ON THE PRUNUS-PLATANUS-ROBINIA REACTION. [In this table PbAc = $Pb(C_2H_3O_2)_2$]

Control tests:		Tests for effect of H_2S on reaction:	
Prunus (Normal) + Robinia (Normal)	= 3	Prunus (Normal + H_2S) + Robinia (Normal + H_2S)	= 3
" " + Platanus "	= 3	" " " + Platanus " "	= 3
Platanus " + Robinia "	= 0	Platanus " " + Robinia " "	= 0

		Prunus (Normal)	Platanus (Normal)	Robinia (Normal)
Robinia	Alk.PbAc filtrate, pH 5.2, Molisch test: trace	3	0	0
	Alk.PbAc precipitate, pH 4.8, Molisch test very strong	0	0	0
	Control (Normal extract)	3	0	0
Platanus	Alk.PbAc filtrate, pH 5.3, Molisch test very weak	3	0	0
	Alk.PbAc precipitate, pH 4.7, Molisch test very strong	0	0	0
	Control (Normal extract)	3	0	0
Prunus	Alk.PbAc filtrate, pH 5.6, 5.5, Molisch test weak	0,0	0,0	0,0
	Alk.PbAc precipitate, pH 5.0, 5.8, Molisch test very strong	0,0	0,0	0,0
	Neutral PbAc filtrate	0	0	0
	Neutral PbAc precipitate	0	3	3
	Control (Normal extract)	0	3	3

This experiment is of interest in distinguishing the reactive components of *Prunus* as opposed to those of *Platanus* and *Robinia*. The former are precipitated by neutral $Pb(C_2H_3O_2)_2$, the latter by neither neutral nor alkaline $Pb(C_2H_3O_2)_2$. The reaction appeared in full strength in a fraction showing only a trace of Molisch reaction and hence could not conceivably be due to a Molisch-carbohydrate in *Platanus* or *Robinia*. The fact that the active constituent of *Prunus* was precipitated by neutral $Pb(C_2H_3O_2)_2$ does not shed much light upon its nature, since this precipitate contains a highly heterogeneous mixture of substances, but it is of interest in view of the later findings regarding its oxalate nature, since many oxalates are precipitated but reclaimable by this technique.

H. Effect of Enzyme Digestion, Contamination, and Ageing on the Reactions

Of considerable interest is the question of whether the reactive ingredients of these extracts are affected by the action of enzymes. Indeed the failures of enzymes, suitably chosen and tested, to inactivate such reactive principles is very strong evidence against their protein nature. Accordingly the following experiments were planned to test this matter.

In the first experiment on this head, *Prunus, Platanus,* and *Robinia* were again used. Normal extracts of each of these species were titrated with KOH to pH 9.0-9.5 and then divided each into two portions. One portion of each was treated with a few drops of Difco Bacto-trypsin. Both portions of each were then covered with toluol and incubated at 37°C. Each day a few drops of the trypsin were added to each experimental tube. The incubation continued for 9 days. During this time no additional alkali was needed, as the rise in pH due to tryptic digestion was not over pH 1 during the experiment. That slight rise in pH which did take place was during the first three days alone. On removing from the oven the extracts all showed a greater or less precipitate, and this precipitate, for the most part, was not brought back into solution on acidification. The extracts were then acidified with HCl to their original pH, filtered or centrifuged, and used in testing. The results of these tests are shown in Table XVIII-A. Where two values are given for any given reaction (as "4, 4") this indicates two separate performances of the experiment, with extracts separately handled throughout. In the table "normal" signifies fresh, unheated extract, "control" signifies extracts titrated and incubated but not trypsinized.

As a second part to this experiment, 15 cc. each of normal extracts of the same three species were slightly acidified and treated with crystalline pepsin. The treated and control tubes were incubated at 37°C. for 8 days and then tested (Table XVIII-B).

For a third part to the same experiment, extracts of the same species were titrated to neutrality with KOH, placed in fermentation tubes at room temperature, and inoculated with baker's yeast. Control tubes remained beside them at room temperature. After five days all were cleared and tested. There was no gas production in any case. The results, (Table XVIII-C) being a duplication of those in part B, are not given in tabular form.

An examination of Table XVIII-A shows that in no case did the trypsin digestion result in a loss of reaction in any one of the three types of extract. The minor variations are not considered of signifi-

cance in view of the decided strength of all the positive reactions. The same is true of the trypsinized extracts (B) and of the extracts treated with yeast. One may conclude from this experiment that the reactive substances are highly resistant to the action of proteolytic enzymes and of the zymase complex, and accordingly the view of their non-protein nature is strengthened.

TABLE XVIII.

EFFECT OF ENZYME DIGESTION ON PRUNUS-PLATANUS-ROBINIA PRECIPITIN REACTION.

A. Effect of trypsin digestion.

		Prunus			Platanus			Robinia		
		Normal	Control	Exper.	Normal	Control	Exper.	Normal	Control	Exper.
Prunus	Normal	0	0	0	4	4	4,4	3	3	3,3
	Control	0	0	0	3	2	2	1	0	0
	Exper.	0	0	0	4,4	4	4,4	3,3	2	2,3
Platanus	Normal	4	3	4,4	0	0	0	0	0	0
	Control	4	2	4	0	0	0	0	0	0
	Exper.	4,4	2	4,4	0	0	0	0	0	0
Robinia	Normal	3	1	3,3	0	0	0	0	0	0
	Control	3	0	2	0	0	0	0	0	0
	Exper.	3,3	0	2,3	0	0	0	0	0	0

B. Effect of pepsin digestion.

Prunus....Normal	0	4	4
Prunus....Treated	0	4	4
Robinia...Normal	4	0	0
Robinia...Treated	4	0	0
Platanus..Normal	4	0	0
Platanus..Treated	4	0	0

C. Effect of yeast digestion. (Omitted. Results exactly as in "B" above)

Beside this experiment in enzyme digestion certain other evidence may be cited relative to the same question. Frequently extracts kept for long periods of time, particularly at room temperature or without protection with toluol, become contaminated with various types of microorganisms. Incidentally such extracts have frequently been reclarified and found still to retain strong precipitin potency. However, in order to have definite data to meet this question an experiment was arranged to illustrate the effect of contaminations on the extracts. In this experiment extracts of 6 species of *Nicotiana* were employed, all of which had become contaminated 3 days after they had been first prepared and tested. The contaminated extracts were cleared and tested again against the same solutions as had been used with the uncontaminated ones three days previously. The results of these tests are given in Table XIX.

It will be seen from an examination of this table that the contamination exerted no inhibitory effect upon the reactive substances, as there is no significant difference in the results of the tests before and after contamination. It should be noted here that the *Nicotiana* species all contained excess calcium and that they were tested against potassium oxalate simply because this gives an accurate measure of the calcium oxalate reaction. The significance of the test in no wise differs from that if *Prunus* had been used in place of the $K_2C_2O_4$. The tests against *N. Rusbyi*, on the other hand, are not due to the calcium oxalate reaction, as will be explained in the following sections, and for this reason the evidence on contamination of this experiment signifies the absence of its deleterious effect not only in the calcium oxalate reaction but in the MN reaction as well.

In addition to the experiments on enzyme digestion and on contamination, one other type of evidence should be considered in this section, namely the influence of age of the extracts. The question has seriously concerned the writers as to whether it was safe to use an extract, however clear, which had been preserved by cold and toluol for long periods of time. Accordingly an experiment to study the effect of age on the extracts was carried out.

A group of extracts (See Table XX) which had all been extracted and preserved at least four months were decanted and tested as is shown in the following table (Table XX).

An examination of the data shows that in no case was there any significant change attributable to the long period of storage. This fact indicates that future workers in this field may with confidence store extracts, at least of this type, for considerable periods of time at 2°C. and covered with toluol, and also that the auto-precipitations occurring in such stored extracts do not weaken the reactions.

I. Effect of Double Precipitation on the Reactions

A final point to consider in studying the analogy between the plant reactions and those of animals is the phenomenon of double precipitation. In serology the specificity of the precipitin reaction is such that if an antiserum A is prepared immune to two antigenic sera B and C, then A may be fully precipitated by B, cleared, and subsequently strongly precipitated by C. Presence of such a phenomenon in plants would strengthen the view that the plant reactions are homologous to those of animals, and absence of this phenomenon would reduce the plant reactions to a relatively simple type of precipitation.

In order to test this point, an experiment was planned involving

TABLE XIX.

EFFECT OF CONTAMINATION ON THE CALCIUM OXALATE AND MN REACTIONS. CON.: CONTAMINATED. UNCON.: UNCONTAMINATED.

	Precipitin reaction against the following species of Nicotiana:											
	alata		Langsdorffii		acuminata		Cavanillesii		sylvestris		glauca	
	Cont.	Uncon.	Con.	Uncon.	Con.	Uncon.	Con.	Uncon.	Con.	Uncon.	Con.	Uncon.
Nicotiana Rustica (M)	2	2	1	1	t	t	1	2	1	1	2	1
Potassium oxalate (M/100)	3	3	3	3	3	3	3	4	3	4	4	3

TABLE XX.

EFFECT OF STORING EXTRACTS FOR LONG PERIODS OF TIME IN THE COLD.

"a" = Test when extracted. "b" = Test after four months of storage.

		Prunus Armeniaca Mikado		Prunus Armeniaca Angu		Photinia villosa		Deutzia scabra plena		Spiraea virginiana serrulata	
		"a"	"b"	"a"	"b"	"a"	"b"	"a"	"b"	"a"	"b"
Hydrangea panic.grfl.	"a"	2		2		0		0			
	"b"		2		2		0		0		
Photinia villosa	"a"	2		2		0					
	"b"		2		2		0				
Deutzia scabra pl.	"a"	t		t				0			
	"b"		1		1				0		
Pyracantha coccinea	"a"	2		2		0				0	
	"b"		2		2		0				0
Spiraea virg.serrul.	"a"	2		2						0	
	"b"		2		2						0

TABLE XXI.

DOUBLE PRECIPITATION IN THE PRUNUS-PLATANUS-ROBINIA-RIBES-HYDRANGEA REACTION COMPLEX.

	Tested against normal extracts of:			
	Robinia	Platanus	Ribes	Hydrangea
Prunus control (Prunus : water = 1 : 4)....	2	2	3	3
Prunus + Robinia. Cleared mixture, 1 : 4...	0	0	0	0
Prunus + Platanus. Cleared mixture, 1 : 4...	0	0	0	0
Prunus + Ribes. Cleared mixture, 1 : 4...	0	0	0	0
Prunus + Hydrangea. Cleared mix., 1 : 4...	0	0	0	0
Robinia (Normal).....	0	0	0	0
Platanus (Normal).....	0	0	0	0
Hydrangea (Normal).....	0	0	0	0
Ribes (Normal).....	0	0	0	0

normal extracts of *Robinia, Platanus, Ribes, Hydrangea,* and *Prunus. Prunus* is positive to the other four, which latter four are negative *inter se.* As the *Prunus* control was prepared an extract consisting of one part normal *Prunus* extract + four parts water. The other extracts were prepared by mixing one part of normal *Prunus* extract with four parts of *Robinia, Ribes, Platanus,* or *Hydrangea* extracts respectively. A voluminous precipitate resulted in all cases. The latter was removed by centrifuging and the resulting cleared mixtures were tested as in Table XXI.

A consideration of these results shows that complete precipitation with an excess of any one of the four counter extracts completely eliminated any further reactivity of the *Prunus* with the other extracts. This is a situation very different from that of animal immunology, and points definitely to a qualitative difference between the plant and animal reactions. Indeed one is assured by a study of Table XXI that the reactive principle in *Prunus* is a single substance fully precipitable by any one of the other four extracts, which latter might well be assumed to contain the same reactive ingredient.

We have now reviewed the evidence regarding the analogy of the *Prunus-Platanus-Ribes-Robinia-Hydrangea* reactions to the zoöimmunitary precipitin reaction. Before passing on to the next subject it would be well briefly to enumerate the points demonstrated in the present section.

A. The reactive substances of the *Prunus-Platanus-Ribes-Robinia* complex differ from the specific protein reactions of animal sera in the following circumstances:
   1. The reactive substance of *Prunus* is manifestly different from that of *Platanus, Robinia, Ribes,* and *Hydrangea.* The reactive substances in these last four species, however, appear to be identical.
   2. The reactive substances in all extracts are highly resistant to heat, alcohol coagulation, variations in pH, enzyme digestion, coagulation by strong acids and alkalies, and bacterial and fungus contaminations.
   3. The reactive substances in all these extracts pass freely through dialyzing membranes impermeable to formed protein but permeable to crystalloids.
   4. There is no evidence of a zone phenomenon in these extracts comparable to that of animal serology.

B. The reactive substances are non-lipoid in nature.

C. They are, at least in some cases, non-carbohydrate in nature.

D. They are unaffected by any of Silberschmidt's various techniques of pre-extraction and solution, including pre-extraction with alcohol, ether, and chloroform, and solution in water, physiological saline (acid or neutral), and N/20 NaOH.

IV. PROOF OF THE CALCIUM OXALATE REACTION

The preceding section having treated with a comparison of the plant precipitin reaction with that of animal immunology and having dealt with the evidence that the plant reaction is non-protein, non-lipoid, and non-carbohydrate, the present section will concern itself with a proof of the exact nature of the reaction in the *Prunus-Platanus-Robinia-Ribes* complex and an extension of this proof to a variety of other plants.

The first successful line of attack of this problem was in experiments in ignition of extracts and precipitates. It has already been pointed out (page 125) that a preliminary experiment in the ignition of extracts failed to reveal the reactive substances in solutions of the ash. However, a new type of experiment was resorted to in order to determine whether the precipitate itself was of organic or inorganic nature.

Clear normal extracts were prepared of *Prunus, Platanus, Robinia,* and *Ribes* in the ordinary way. These were centrifuged for several minutes to throw down any further organic débris. Then the following mixtures were placed in centrifuge tubes:

Prunus (5 cc.) + *Platanus* (5 cc.)
" " + *Robinia* (5 cc.)
" " + *Ribes* (5 cc.)

These were permitted to interact for 10 minutes, and were then centrifuged. Each precipitate from centrifuging was washed in three changes of distilled water, suspending the precipitate in water and centrifuging after each change. The precipitates were then suspended in the last water and each was ignited in a small beaker at dull red heat (500°C.). In no case was there evidence of any appreciable amount of carbon in the process of ignition. The deposits remained perfectly white throughout, with only the slightest trace of pale brown momentarily appearing. This experiment was subsequently repeated several times but always with the same result.

A second line of attack was the solubilities of the precipitates. It was found that these were highly insoluble in all ordinary solvents. In fact solution was successful only in strong acid, while alcohol, lipoid solvents, boiling water, benzol and toluol, weak acids, and alkalies all failed to dissolve them.

At this point qualitative analysis of the precipitates was resorted to. The analysis gave the following results:

1. In physical appearance the precipitates resembled organic solids in being limey, granular, heavy, and easily centrifuged.
2. Qualitative analysis showed calcium and oxalate ions to be the only ones present in any appreciable quantity.
3. Microscopically the precipitates were in the form of regular granules, not amorphous, identical in appearance with certain commercial samples of calcium oxalate.
4. Recrystallization of the precipitates (by solution in 6N H_2SO_4 followed by precipitation by neutralization with 4N KOH) gave an abundance of crystals of the characteristic size and shape of CaC_2O_4 crystals and indistinguishable from crystals of a commercial sample of CaC_2O_4 similarly treated.
5. Treatment of the granules of the precipitates with 6N H_2SO_4 under the microscope showed first a moderate solution followed by a very striking conversion of the remainder of the granules into the characteristic raphides of $CaSO_4$. This is a fairly accurate test for CaC_2O_4, and was precisely the behavior of a sample of commercial CaC_2O_4 similarly treated.
6. When alcohol was added to the H_2SO_4 solution of the precipitates, there was a precipitation (test for calcium).
7. The acid solution of the precipitates strongly reduced potassium permanganate (test for oxalates).

Next, through the courtesy of A. D. Bliss of the Harvard chemistry laboratories, a quantitative analysis was made of specimens of the washed precipitate prepared as was that in the ignition experiments described above. Mr. Bliss' analysis of the precipitate gave the following results.

The preliminary identification of the precipitate as calcium oxalate on the basis of its qualitative reactions, etc., was well confirmed by the quantitative analytical data, for in view of the stoichiometric relationships involved nothing else could be present to any great extent. The small amount of substance available naturally rendered extensive analyses impossible, but the calcium content was determined and also the permanganate-reducing power of the organic radical involved.

To determine the calcium, a specimen was weighed, ignited at red heat, then cooled and weighed. Calculating the weight of calcium oxalate equivalent to the calcium oxide obtained, allowing for the fact that when prepared from wet materials and in contact with moist air calcium oxalate exists as the monohydrate, $CaC_2O_4.H_2O$, the result was 85.89% of calcium oxalate in the material.

Since the calcium oxalate might not be pure, it was next dissolved in HCl and the calcium precipitated with ammonium oxalate in ammoniacal solution. The calcium oxalate was filtered off, washed, and ignited to constant weight as the oxide. The product was pure white, as it should have been. With the weight of calcium oxalate thus obtained the calculated amount of calcium oxalate was 84.61%. This percentage does not differ very much from the first, showing that the oxide from the first ignition was practically pure calcium oxide.

The percentage of calcium in the original sample was then found to be 23.22% as compared with 27.43% in pure calcium oxalate. This discrepancy may have been due to one or more of several causes: (1) presence of metals other than calcium, (2) other organic aids than oxalic, of higher molecular weight, or (3) organic matter which would disappear on ignition.

In order to check the results of the calcium determination, a titration of the reducing power of the material was made with standard permanganate solution. This titration gave for the percentage of calcium oxalate in the precipitate 87.94%. The difference between this value, 87.94%, and that found from the calcium analysis, 84.61% may have been due in part to the normal errors of analysis, to a possible difference in composition of the material, since the two samples were independently obtained, and lastly to higher molecular weight acid radicals or organic débris in the sample, as mentioned above.

The qualitative and quantitative analyses having agreed as to the calcium oxalate nature of the precipitates from the *Prunus-Platanus-Robinia-Ribes* reactions, the next step was to determine the behavior of extracts of these and other plants in the presence of solutions of calcium and oxalate. The first test of this type was with normal extracts of *Prunus, Platanus,* and *Robinia* as tested against one another and against certain calcium compounds as well as certain oxalates. These tests were all performed in the customary manner, and the results are given in the following table (Table XXII).

A study of this table shows that *Prunus* behaves exactly as do the oxalates, while *Platanus* and *Robinia* behave in the same fashion as the calcium salts.

This same type of analysis was next applied to a variety of all the woody plants which were at the time available. All were tested against potassium and ammonium oxalate and calcium chloride and nitrate as well as against one another (Table XXIII). All the plants were found to fall into two groups, one group including only *Prunus* which was negative to the oxalates and positive to the calcium salts, the other

TABLE XXII.

RELATION OF THE PRUNUS-ROBINIA-RIBES REACTION TO THE PRESENCE AND ABSENCE OF CALCIUM AND OXALATE.

	Parts soluble in 100 parts of water:	Ribes, normal extract:	Robinia, normal extract:	Prunus, normal extract:	Potassium oxalate:	Ammonium oxalate:
Calcium nitrate	93.	0	0	2	2	2
Calcium chloride	70.	0	0	2	2	2
Calcium acetate	42.	0	0	2	2	2
Calcium sulphate	.2	0	0	1	1	1
Calcium tungstate	.2	0	0	0	0	0
Calcium phosphate	Trace	0	0	0	0	0
Calcium fluoride	Trace	0	0	0	0	0
Potassium oxalate	33.	2	2	0	0	0
Ammonium oxalate	4.2	2	2	0	0	0

TABLE XXIII.

PRESENCE OR ABSENCE OF PRECIPITIN REACTIONS IN CERTAIN WOODY PLANTS IN RELATION TO PRESENCE OR ABSENCE OF CALCIUM AND OXALATE.

	Potassium oxalate	Ammonium oxalate	Prunus Armeniaca	Platanus acerifolia	Robinia fertilis	Ribes Carrieri	Hydrangea paniculata	Syringa vulgaris	Ligustrum obtusifolium	L. vulgare	L. ibota	Calcium chloride	Calcium nitrate
Potassium oxalate	0	0	0	+	+	+	+	+	+	+	+	+	+
Ammonium oxalate	0	0	0	+	+	+	+	+	+	+	+	+	+
Prunus Armeniaca	0	0	0	+	+	+	+	+	+	+	+	+	+
Platanus acerifol.	+	+	+	0	0	0	0	0	0	0	0	0	0
Robinia fertilis	+	+	+	0	0	0	0	0	0	0	0	0	0
Ribes Carrieri	+	+	+	0	0	0	0	0	0	0	0	0	0
Hydrangea panicul.	+	+	+	0	0	0	0	0	0	0	0	0	0
Syringa vulgaris	+	+	+	0	0	0	0	0	0	0	0	0	0
Lig. obtusifolium	+	+	+	0	0	0	0	0	0	0	0	0	0
L. vulgare	+	+	+	0	0	0	0	0	0	0	0	0	0
L. ibota	+	+	+	0	0	0	0	0	0	0	0	0	0
Calcium chloride	+	+	+	0	0	0	0	0	0	0	0	0	0
Calcium nitrate	+	+	+	0	0	0	0	0	0	0	0	0	0

group containing the remainder of the woody species, which were perfectly negative *inter se* and to the calcium salts but all positive to the oxalates and to *Prunus*.

We thus see that all of the reactions observed in the woody plants are susceptible to explanation on the basis of a calcium oxalate precipitation occurring as a result of the interaction of oxalate ions in *Prunus* with calcium ions in the remainder of the extracts. No other hypothesis is necessary to explain any of the reactions in this group of plants, and that the reactions observed are due to calcium oxalate is manifest not only from the analytical studies above but also from the simple chemical fact that the tests of these species with calcium salts and with oxalates (Table XXIII) demonstrates that they must contain calcium or oxalate as indicated. Furthermore it follows from the chemistry of such ions that if one adds a solution containing free calcium to one containing free oxalate, a precipitate must result. We thus find that not only *do* these precipitations occur between the extracts under consideration, but that they *must* occur from the chemical laws governing the behavior of calcium in the presence of an oxalate.

It was now felt desirable to extend these results to a greater variety of plants to observe the extent of distribution and the importance of the calcium oxalate reaction in plants, particularly in those in which much of the work on the precipitin reaction has been accomplished. Accordingly the same type of experiment as that of Table XXIII was applied to a variety of species of the *Solanaceae*. The results of these tests are gathered and arranged in Table XXIV.

A glance at this table will assure one that the same factors are at work here as were observable in the woody plants. Of the 21 species chosen at random, 8 were negative to the oxalates and positive to the calcium salts, 10 others were negative to the calcium salts and positive to the oxalates. The 8 "oxalate" extracts were all in varying degree positive to the 10 "calcium" extracts. Three extracts, *Cyphomandra, Browallia,* and *Datura Wrightii,* were negative or practically so to both salts, and were correspondingly negative to both the "calcium" and "oxalate" extracts. Moreover the "oxalate" extracts were perfectly negative *inter se,* while the "calcium" extracts were nearly so. Thus the same remarks as were applied to the results within the woody plants under consideration apply equally to the *Solanaceae*. The calcium oxalate reaction is of frequent occurrence in the *Solanaceae* as it was in the woody plants, almost all of the reactions in Table XXIV being explainable in terms of calcium oxalate precipitations, and moreover the behavior of these extracts with the pure solutions of the salts requires that

such "precipitin reactions" take place when the various pairs of extracts are combined.

In observing the results in Table XXIV it will be seen that the "calcium" extracts are not perfectly negative *inter se*. However, the few reactions which do appear in this block of the table will be explained in the following section.

TABLE XXIV.

PRECIPITIN REACTIONS AND CALCIUM OXALATE REACTIONS OF CERTAIN SOLANACEAE.

		Ammonium oxalate .02N	Potassium oxalate .01N	Atropa Belladonna	Datura ferox	Solanum tuberosum	Capsicum frutescens	Datura metel	D. innoxia	Physalis Peruviana	Salpiglossis sinuata	Cyphomandra betacea	Browallia viscosa	Datura Wrightii	Nicotiana Rusbyi	N. paniculata	Petunia violacea	Lycopersicum cerasiforme	Solanum Capsicastrum	Nicotiana rustica	N. suaveolens	N. Tabacum	Solanum nigrum	S. melongena	Calcium chloride .005N	Calcium nitrate .005N
	Ammonium oxalate .02N	0	0	0	0	0	0	0	0	0	0	0	0	0	t	t	1	2	1	2	2	2	2	2	1	1
	Potassium oxalate .01N	0	0	0	0	0	0	0	0	0	0	0	t	t	1	2	1	1	2	2	2	2	2	3	2	2
	Atropa Belladonna	0	0	0	0	0	0	0	0	0	0	0	t	0	2	3	3	3	2	3	3	3	3	3	3	3
	Datura ferox	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	3	2	2	2	3	2	2	2	2	3
	Solanum tuberosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	2	4	2	3	2	2	1	2
Ox	Capsicum frutescens	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	2	2	1	3	2	3	3	2	2	1
	Datura metel	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	2	1	3	2	3	1	1	1	2
	Datura innoxia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	3	1	2	3	2	1	1	1	1
	Physalis Peruviana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	2	2	2	2	2	2	1	1	1
	Salpiglossis sinuata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	1	2	0	0	1	1
	Cyphomandra betacea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	t	t	t	0	0	0	0
	Browallia viscosa	0	t	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Datura Wrightii	0	t	0	0	0	0	0	0	0	0	0	0	0	t	t	0	t	0	0	0	t	0	0	0	0
	Nicotiana Rusbyi	t	1	2	0	0	2	0	0	0	0	0	0	t	0	0	0	0	1	1	1	1	0	t	0	0
	N. paniculata	t	2	3	3	2	3	3	3	2	0	0	0	t	0	0	0	0	0	0	0	0	0	0	0	0
	Petunia violacea	1	1	3	3	2	2	2	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Lycopersicum cerasiforme	2	1	3	2	1	2	2	3	2	t	0	0	t	0	0	0	0	0	0	0	0	0	0	0	0
	Solanum Capsicastrum	1	2	2	2	2	1	1	1	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Ca	Nicotiana rustica	2	2	3	2	4	3	3	2	2	2	t	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	N. suaveolens	2	2	3	3	2	2	2	3	2	1	t	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	N. Tobacum	2	2	3	2	3	3	3	2	2	2	t	0	t	1	0	0	0	0	0	0	0	0	0	0	0
	Solanum nigrum	2	2	3	2	2	3	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	S. melongena	2	3	3	2	2	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Calcium chloride .005N	1	2	3	2	1	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Calcium nitrate .005N	1	2	3	3	2	1	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

We may thus conclude this section with the following brief statement of the occurrence of the calcium oxalate reaction: The reactions between *Prunus*, *Platanus*, *Robinia*, and *Ribes* have been proved to be due to the presence in *Prunus* of free oxalate which combines with free calcium in the other extracts to produce a precipitation of calcium oxalate. The existence of free calcium and free oxalate in various other

woody plants as well as in a variety of the *Solanaceae* has been demonstrated, and as a necessary corollary, calcium oxalate precipitations must occur whenever two extracts containing these two ions respectively are mixed. Indeed all the precipitations observed in the preceding tables in the woody plants and nearly all in the *Solanaceae* were precipitates of calcium oxalate.

Mentally reviewing the evidence on the nature of the reaction presented in the earlier part of this paper, all that has been obtained is in perfect agreement with this conception, the relative insensitivity to pH, heat, enzymes, and alcohol, the effect of increasing salt concentration, the regular behavior in dilution, the readiness with which the reactive principles pass through dialyzing membranes, their insolubility in alcohol and lipoid solvents, and their identity in the experiments in double precipitation, all are in agreement with the calcium oxalate interpretation.

However, one very important question remains: Is the calcium oxalate reaction sufficient to account for *all* of the reactions observed? The answer to this question will be considered in the following section.

V. PROOF OF THE EXISTENCE OF OTHER REACTIONS

At this stage in the development of the problem, it was felt that a very important confirmation of the calcium oxalate explanation would result from the behavior of the "calcium" woody extracts in the presence of the "calcium" solanaceous extracts. The number of *Solanaceae* was increased to 36 species for this experiment, and the precipitin reactions of all the possible combinations of these 42 species were determined. In addition, all the extracts were tested against two oxalates and against two calcium salts. The species were then arranged according to their reactivity with the salts and tabulated as in Table XXV.

Again the 42 species fall into three classes, a "calcium" class, an "oxalate" class, and a class reactive to neither. The "oxalate" class (Ca—, Ox+) is negative *inter se,* as would have been predicted; so also is the (Ca—, Ox—) class. The "calcium" class (Ca+, Ox—) are all positive to the Ca—, Ox+) class and reciprocally. Thus far the calcium oxalate reaction is perfectly sufficient for our explanation. But when one concentrates on the inter-reactions of the members of the (Ca+, Ox—) class there are many positive reactions unaccounted for. These, however, are not irregularly distributed through the table but appear to be the reactions with certain specific extracts, notably *Robinia, Platanus,* and *Ribes.* Moreover the strength of reaction of any given solanaceous extract is proportional toward all of the three

woody extracts mentioned. It is thus clear that there is another reaction present, occurring between the woody species and the majority of the *Solanaceae*. This reaction can be studied best by eliminating

TABLE XXV.

A COMPREHENSIVE TABLE OF ALL THE PRECIPITIN REACTIONS IN THE PLANTS EMPLOYED IN THIS STUDY, EMPHASIZING PARTICULARLY THE CALCIUM OXALATE REACTION.

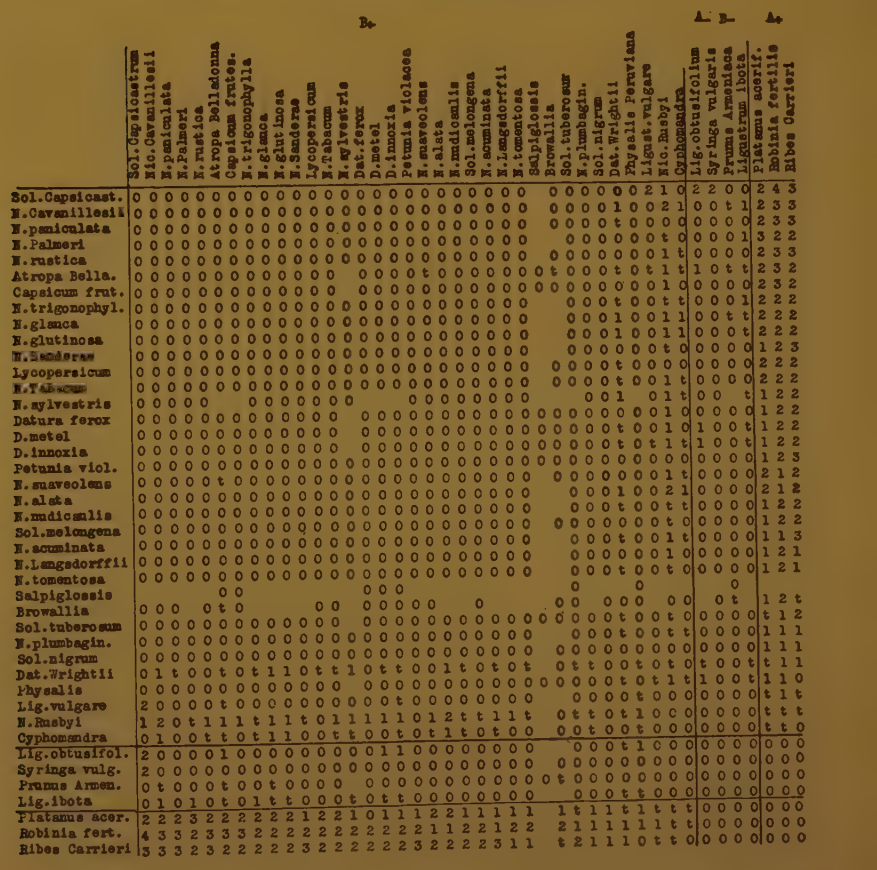
	Ca- Ox+									CaOx-			Ca+ Ox-																													
	Atropa Bella.	Dat.ferox	Prunus	Sol.tuberos.	Capsicum	Dat.metel	Physalis	Dat.innoxia	Salpiglossis	Cyphomandra	D.Wrightii	Browallia	Lig.vulgare	L.obtusifol.	Nic.Rusbyi	Petunia	Robinia	Platanus	Ribes	N.paniculata	N.Palmeri	N.trigonoph.	N.Sanderae	Lycopersicum	Sol.Capsic.	N.plumbagin.	Lig.ibota	Syringa	Sol.nigrum	N.suaveolens	N.alata	N.acuminata	N.glauca	N.glutinosa	N.nudicaulis	N.Langsdorff.	N.tomentosa	N.Tabacum	N.Cavanill.	N.rustica	N.sylvestris	Sol.melongena
Atropa	0	0	t	0	0	0	0	0	0	0	0	t	1	1	2	3	3	2	2	3	1	1	1	3	2	2	1	2	3	3	3	1	3	2	2	1	1	3	2	3	2	3
Datura ferox	0	0	t	0	0	0	0	0	0	0	0	0	1	1	0	3	2	2	2	3	2	1	1	2	2	2	1	2	2	3	3	2	2	2	2	1	2	2	3	2	2	2
Prunus Armen.	t	t	0	0	0	0	0	0	0	0	t	t	2	2	1	2	2	1	1	3	t	1	2	2	2	1	2	3	2	3	2	3	2	2	2	2	1	4	3	3	2	2
Sol.tuberos.	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	2	1	2	3	2	2	2	1	1	2	1	1	2	2	2	3	1	3	2	3	1	2	3	2	4	2	2
Capsicum	0	0	0	0	0	0	0	0	0	0	0	0	t	1	2	2	2	2	2	3	1	t	1	2	1	t	1	1	3	2	2	1	2	2	2	t	1	3	1	3	2	2
Dat.metel	0	0	0	0	0	0	0	0	0	0	0	0	1	t	0	2	2	2	2	3	1	2	1	2	1	1	1	1	1	2	2	2	2	2	3	1	1	3	1	3	2	1
Physalis	0	0	0	0	0	0	0	0	0	0	0	0	t	1	0	1	1	2	1	2	1	1	1	2	2	1	2	1	2	2	2	1	2	2	2	2	2	2	2	2	2	1
Dat.innoxia	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	2	1	2	2	3	1	1	1	3	1	1	t	1	1	3	2	2	2	2	2	1	1	2	1	2	1	1
Salpiglossis	0	0	0	0	0	0	0	0	0	0	0	0			0	1	1	2	1	0				0	0			0	0	1								2		2		0
Cyphomandra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	t	t	0	0	0	t	0	0	0	t	0	0	0	t	1	t	1	1	t	0	0	t	1	t	t	0
Dat.Wrightii	0	0	t	0	0	0	0	0	0	0	0	0	0	0	t	0	1	t	t	t	0	t	0	t	0	t	t	0	0	0	1	t	1	1	t	0	t	t	1	0	1	0
Browallia	t	0	t	0	0	0	0	0	0	0	0	0			0	0	2	1	t	0				0	0			0	0	0								0		0		0
Lig.vulgare	1	1	2	1	t	1	t	1		0	0		0	0	0	0	1	t	t	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L.obtusifol.	1	1	2	1	1	t	1	2		0	t		0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nic.Rusbyi	2	0	1	0	2	0	0	0	0	0	t	0	0	0	0	0	t	t	t	0	t	t	t	0	1	t	0	0	0	1	2	t	1	1	t	1	t	1	2	1	1	t
Petunia	3	3	2	2	2	2	1	2	1	0	0	0	0	0	0	0	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Robinia	3	2	2	1	2	2	1	1	1	t	1	2	1	0	t	2	0	0	0	3	2	2	3	2	4	1	0	0	1	1	1	1	2	2	2	2	2	2	3	2	2	1
Platanus	2	2	1	2	2	2	2	2	2	t	t	1	t	0	t	1	0	0	0	2	3	2	1	2	2	1	0	0	1	2	2	1	2	2	1	1	1	2	2	2	1	1
Ribes Carr.	2	2	1	3	2	2	1	2	1	0	1	t	t	0	t	2	0	0	0	3	2	2	2	2	3	1	0	0	1	2	2	3	2	2	2	1	1	2	3	3	2	2
N.paniculata	3	3	3	2	3	3	2	3	0	0	t	0	0	0	0	0	3	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Palmeri	1	2	t	2	1	1	1	1		0	0		0	0	t	0	2	3	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.trigonoph.	1	1	1	2	t	2	1	1		t	t		0	0	t	0	2	2	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Sanderae	1	1	2	1	1	1	1	1		0	0		0	0	t	0	3	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lycopersicum	3	2	2	1	2	2	2	3	0	0	t	0	0	0	0	0	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sol.Capsic.	2	2	2	2	1	1	2	1	0	0	0	0	2	2	1	0	4	2	3	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.plumbagin.	2	2	1	1	t	1	1	1		t	t		0	0	t	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lig.ibota	1	1	2	1	1	1	2	t		0	t		0	0	0	0	0	0	0	0	1	1	0	0	2	0	0	0	0	0	0	0	t	t	0	0	0	0	1	0	t	0
Syringa vulg.	2	2	3	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sol.nigrum	3	2	2	2	3	1	2	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.suaveolens	3	3	3	2	2	2	2	3	1	t	0	0	0	0	1	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.alata	3	3	2	3	2	2	2	2		1	1		0	0	2	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.acuminata	1	2	2	1	1	2	1	2		t	t		0	0	t	0	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.glauca	3	2	2	3	2	2	2	2		1	1		0	0	1	0	2	2	2	0	0	0	0	0	0	0	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.glutinosa	2	2	2	2	2	2	2	2		1	1		0	0	1	0	2	2	2	0	0	0	0	0	0	0	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.nudicaulis	2	2	2	3	2	3	2	2		t	t		0	0	t	0	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Langsdorff.	1	1	2	1	t	1	2	1		0	0		0	0	1	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.tomentosa	1	2	1	2	1	1	2	1		0	t		0	0	t	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Tabacum	3	2	4	3	3	3	2	2	2	t	t	0	0	0	1	0	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Cavanill.	2	3	3	2	1	1	2	1		1	1		0	0	2	0	3	2	3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.rustica	3	2	3	4	3	3	2	2	2	t	0	0	0	0	1	0	2	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.sylvestris	2	2	2	2	2	2	2	1		t	1		0	0	1	0	2	1	2	0	0	0	0	0	0	0	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sol.melongena	3	2	2	2	2	1	1	1	0	0	0	0	0	0	t	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Calc.chloride	3	2	2	1	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Calc.nitrate	3	3	2	2	1	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Potas.oxalate	0	0	0	0	0	0	0	0	0	0	t	t	1	1	1	1	1	1	1	2	2	2	2	1	2	2	1	2	2	2	3	3	3	3	3	3	3	2	4	2	4	3
Ammon.oxalate	0	0	0	0	0	0	0	0	0	0	0	0	t	t	t	1	1	1	1	t	t	t	t	2	1	1	t	2	2	2	1	1	1	1	1	1	1	2	t	2	1	2

entirely all of the calcium oxalate reactions. Such elimination automatically occurs in the interreactions of the (Ca+, Ox—) group and can be made to extend to all of the extracts in Table XXV by adding a slight excess of calcium to the (Ca—, Ox+) extracts and filtering.

Such was done, and the precipitin reactions of all these species were determined anew. They are tabulated in Table XXVI, the arrangement of species being in order of their decreasing reactivity toward

TABLE XXVI.

ALL THE PRECIPITIN REACTIONS IN THE PLANTS STUDIED AFTER ELIMINATION OF THE CALCIUM OXALATE REACTION. EMPHASIZING THE AB REACTION.



Robinia, *Platanus*, and *Ribes*, which latter are plainly similar with regard to this reaction.

We now find that we are able to separate all the extracts into three groups, one group (A—, B+) which is positive toward the three woody species, a second group (A+, B—) which is composed of these three

woody species, and a third group (A—, B—) which is negative to both the former groups. Assuming the existence of two reactive principles, A in *Platanus, Ribes,* and *Robinia,* B in the *Solanaceae,* and neither in the *Oleaceae,* A reacting with B to produce a precipitate, then nearly all of the reactions are accounted for. This AB reaction, the existence of which later experimentation has further confirmed, thus easily accounts for the majority of the reactions not reduceable to calcium oxalate.

A study of Table XXVI but eliminating from consideration the reactions of *Platanus, Robinia,* and *Ribes* is of interest in the following connection. If the calcium oxalate and AB reactions were the only reactions present in this group of extracts, all the remaining combinations should give wholly negative results, since they all lack both oxalate and principle A. Such is nearly but not entirely the case. There are a few reactions remaining, particularly those of *Nicotiana Rusbyi, Datura Wrightii,* and *Cyphomandra.* A reorganization of the data in Table XXVI so as to include only the oxalate-negative, A-negative extracts, and arranged according to their reactivity with *Nicotiana Rusbyi* gives the interesting situation of Table XXVII.

The reactions of *Datura Wrightii* and *Cyphomandra* are plainly correlated with those of *N. Rusbyi,* as possibly are those of *Prunus* and *Ligustrum ibota.* The table clearly points to a third pair of reactive substances, M in the species just mentioned which precipitates in the presence of N, present in most of the *Solanaceae* but absent in *Solanum nigrum, Ligustrum vulgare, Syringa, Petunia, Browallia,* and *Salpiglossis.*

The calcium oxalate, AB, and MN reactions together account for all but a very small percentage of the reactions observed in these species. There still remain, however, a few scattered reactions of which those of *Ligustrum obtusifolium* are most striking. Rearranging, then, the remaining oxalate-negative, A-negative, M-negative extracts as nearly as possible in order of their reactivity with the *Oleaceae,* we obtain Table XXVIII.

Table XXVIII assumes the presence of a reactive principle Y present in the *Oleaceae* considered, lacking in all the other plants, and precipitating in the presence of X which is found in certain of the *Solanaceae* (*Datura metel, D. innoxia, Physalis, Atropa,* and *Solanum Capsicastrum*).

This scheme accounts for every one of the reactions observed in these 42 species of plants. It is, moreover, confirmed by another type of experiment which will be here briefly mentioned.

It will be recalled (page 156) that when *Prunus* extract was thoroughly precipitated with *Platanus*, *Ribes*, *Robinia*, or *Hydrangea*, the resulting clear mixture was perfectly negative to the remaining woody

TABLE XXVII.

PRECIPITIN REACTIONS IN THE PLANTS STUDIED AFTER ELIMINATION OF THE CALCIUM-OXALATE AND AB REACTIONS. EMPHASIZING THE MN REACTION.

	Nic.alata	N.Cavanillesii	N.glauca	N.glutinosa	N.sylvestris	N.Tabacum	Atropa Bella.	N.acuminata	N.rustica	Datura metel	N.trigonoph.	Dat.innoxia	N.suaveolens	Physalis	N.nudicaulis	N.plumbagin.	Sol.Capsic.	Datura ferox	N.Langsdorffii	Capsicum	N.tomentosa	Sol.tuberosum	N.paniculata	N.Palmeri	N.Sanderae	Lycopersicum	Sol.melongena	L.obtusifol.	Sol.nigrum	Lig.vulgare	Syringa vulg.	Petunia viol.	Salpiglossis	Browallia	Prunus Armen.	Lig.ibota	Cyphomandra	Dat.Wrightii	N.Rusbyi
Nic.alata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	1	1	2
N.Cavanillesii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		t	1	1	1	2
N.glauca	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				t	t	1	1	1
N.glutinosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	t	1	1	1
N.sylvestris	0	0	0	0	0	0		0	0		0		0	0	0	0	0		0		0		0	0	0	0	0	0	0	0	0					t	1	1	1
N.Tabacum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	t	t	1
Atropa Bella.	0	0	0	0		0	0	0	0	0	0	0	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	t	0	0	t	t	t	t	t	1
N.acuminata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	t	t	1
N.rustica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	t	0	1
Datura metel	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	t	0	1
N.trigonoph.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	1	t	t	t
Dat.innoxia	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	t	0	0	0	0	0	t	t	1
N.suaveolens	0	0	0	0	0	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	t	0	0	0	0	0	t	t	1
Physalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	t	0	0	0	0	0	t	t	1
N.nudicaulis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	t	t	t
N.plumbagin.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	t	t	t
Sol.Capsic.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	2	0	0	1
Datura ferox	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	1
N.Langsdorffii	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	1
Capsicum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	t	t
N.tomentosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	t	t
Sol.tuberosum	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	t	0
N.paniculata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			0	1	0	0	t
N.Palmeri	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	t
N.Sanderae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	t	0
Lycopersicum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	t
Sol.melongena	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	t	0
L.obtusifol.	0	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Sol.nigrum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Lig.vulgare	0	0	0	0	0	0	t	0	0	0	0	t	0	t	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Syringa vulg.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Petunia viol.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Salpiglossis																																0			0				
Browallia		0			0	t		0	0		0	0	0			0	0		0		0		0	0			0	0		0	0			0	t		0	0	0
Prunus Armen.	0	t	t	0		0	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	t		0	0	0	0
Lig.ibota	0	1	t	t	t	0	t	0	0	t	1	t	0	t	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Cyphomandra	1	1	1	1	t	t	t	t	t	0	t	t	t	t	t	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0
Dat.Wrightii	1	1	1	1	1	t	t	t	0	t	t	t	0	t	t	t	0	0	0	0	t	t	0	0	t	0	0	t	0	0	0	0	0		0	t	0	0	t
N.Rusbyi	2	2	1	1	1	1	1	1	1	1	t	1	1	1	t	t	1	1	1	1	t	t	0	t	t	0	t	0	0	0	0	0	0		0	0	0	t	0

plants. When, on the other hand, *Prunus* was allowed thoroughly to interact with *Ribes*, then cleared, the resulting clear mixture was precipitated strongly by *Petunia*, *Lycopersicum*, *Nicotiana Tabacum*, *N. rustica*, and *N. paniculata*. This accordingly offers additional evidence as to the presence of a second reaction, in this case the AB reaction.

There is, then, no doubt that beside the calcium oxalate reaction there exist certain other reactions in this group of plants. These additional reactions have been tentatively referred to as the AB, MN, and

TABLE XXVIII.

PRECIPITIN REACTIONS IN THE PLANTS STUDIED AFTER ELIMINATION OF THE CALCIUM OXALATE, AB, AND MN REACTIONS. EMPHASIZING THE XY REACTION.

	Y+			XY-																										X+			
	L.obtusifol.	L.vulgare	Syringa vulg.	Browallia	N.suaveolens	N.Cavanillesii	N.glauca	N.glutinosa	N.sylvestris	N.tabacum	N.acuminata	N.rustica	N.trigonophylla	N.alata	N.mudicaulis	N.plumbagin.	N.Langsdorfii	N.tomentosa	N.paniculata	N.Palmeri	N.Sanderae	Capsicum	Lycopersicum	Petunia	Salpiglosis	Datura ferox	Sol.tuberosum	S.melongena	S.nigrum	Datura metel	D.innoxia	Physalis	Atropa	S.capsicastrum
L.obtusifol.	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2
L.vulgare	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	t	t	t	2
Syringa vulg.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Browallia			0	0	0	0				0		0								0			0	0	0		0	0	0	0	0	0	0	0
Nic.suaveol.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Cavanilles.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.glauca	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.glutinosa	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.sylvestris	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0				0			0		0
N.tabacum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.acuminata	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.rustica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.trigonophy.	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.alata	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.mudicaulis	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.plumbagin.	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Langsdorfii	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.tomentosa	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.paniculata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Palmeri	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.Sanderae	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Capsicum	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lycopersicum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Petunia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salpiglossis	0	0																	0		0				0	0	0			0	0	0	0	
Datura ferox	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.tuberosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.melongena	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sol.nigrum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Datura metel	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D.innoxia	1	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Physalis	1	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Atropa	1	t	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.capsicast.	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

XY reactions. What is their nature? It will be the purpose of the following section to answer this question as fully as the experimental data permit, although it must be freely confessed that the investigation in this direction has only begun.

VI. PROPERTIES OF THE AB, MN, AND XY REACTIONS

A. Heat Experiments

The AB reaction, according to our analysis of the preceding section, results from the interaction of a substance A present in *Robinia, Platanus,* and *Ribes* with a substance B present generally in the *Solanaceae* but absent in the *Oleaceae*. The MN reaction results in a precipitation from the interaction of a substance M present particularly in *Nicotiana Rusbyi* and *Datura Wrightii* with a substance N present in many of the other *Solanaceae*.

TABLE XXIX.

EFFECT OF HEAT ON THE REACTIVE SUBSTANCES RESPONSIBLE FOR THE AB REACTION.

A	B	Precipitin reaction of A with B:							
Normal	Heated	Unheated	B heated to:						
extract:	extract:	control(B)	40°	50°	60°	70°	80°	90°	100°
Platanus	N. Rusbyi	1	1	1	1	1	1	1	1
Ribes	N. Rusbyi	2	2	2	2	2	2	2	2
Platanus	N. Tabacum	1	1	1	1	1	1	1	1
Ribes	N. Tabacum	1	1	1	1	1	1	1	1
Platanus	N. rustica	2	2	2	2	2	2	2	2
Ribes	N. rustica	2	2	2	2	2	2	2	2
Platanus	Dat. Wright.	2	2	2	2	2	2	2	2
Ribes	Dat. Wright.	2	2	2	2	2	2	2	2

The first test regarding the nature of the AB reaction was with regard to heat. Fresh leaves of *Nicotiana Rusbyi* (B), *N. rustica* (B), and *Datura Wrightii* (B) were extracted for 18 hours in physiological saline. They were then filtered and the normal extracts were used in the following tests. Normal aqueous extracts of dried leaves of the following were also used: *N. Tabacum* (B), *Platanus* (A), and *Ribes* (A). The solanaceous extracts were then divided each into 8 fractions and the various fractions were heated in water baths for ½ hour intervals at temperatures ranging from 40°C. to boiling. The results of this experiment are given in Table XXIX.

Several considerations develop from a study of this table.

1. The B factor (but not necessarily the A factor as far as we know at this point) is not rendered inactive by heating for ½ hour at temperatures up to 100°C. There was observed in some cases a very slight drop in potency in the extracts heated at temperatures above 58°C., but this was so slight as not to be scoreable. In any case

there was still a reaction approximately equal to the control reaction in all the various heated extracts. There was a heavy precipitation due to heating in all the extracts but *N. Tabacum,* but removal of this precipitate did not influence the reaction.

2. The extracts of *N. Rusbyi* and *D. Wrightii* were tested against *N. Tabacum* and *N. rustica* at the beginning of this experiment, but the results (in contrast with those of Table XXVII) were negative or very weak. Thus the MN reaction is dependent either upon the variability of different plants or on the solvent used in extraction. This applies to both *N. Rusbyi* and *D. Wrightii.* Many subsequent extractions have confirmed this finding: There is considerable variation with regard to the presence and strength of the M principle in experimental plants of the same species. This fact, however, does not apply to the other principles studied thus far.
3. The AB reaction is manifestly different from the MN reaction, in that extracts wholly negative for the one are strongly positive for the other. Both are distinct from the calcium oxalate reaction, since in this experiment the tests with $Ca(NO_3)_2$ remove the possibility that a calcium oxalate reaction could be involved here.

As a further test of the effect of heat on these reactions, normal extracts of *Ribes, Platanus, N. Rusbyi,* and *N. Langsdorffii* were prepared. In this case the *N. Rusbyi* (M) was negative to the *N. Langsdorffii* (N). Each extract was divided into two samples, one sample being retained as a control, the other heated in flowing steam at 0-2 lbs. pressure for 3 hours. The *Ribes* and *Platanus* extracts remained clear throughout the heating, the other two species autoprecipitated and were cleared before testing. The results of the precipitin tests, which were then performed, are given in Table XXX.

It will be seen from this experiment that the AB reaction is unaffected by heating the extracts for 3 hours at 100°C. This applies to both A and B principles.

Further tests of the AB and MN reaction were performed under similar conditions. Active A, B, M, and N extracts were prepared in the customary way, heated and cleared as in the preceding experiment, and tested as in Table XXXI.

Here the results confirm those of the preceding experiment in showing the AB reaction to be coctostable, while there is a weakening of both M and N principles due to the heating. In this respect, then, as well as in others which will be seen later, the AB reaction differentiates itself from the MN reaction. Tests with potassium oxalate demonstrated that the calcium oxalate reaction was not involved here.

TABLE XXX.

EFFECT OF LONG-CONTINUED HEATING ON THE AB REACTION.

	Precipitin reaction with:			
	Nicotiana Rusbyi		N.Langsdorfii	
	Control	Heated	Control	Heated
Ribes.....Control..	2	2	2	2
Ribes.....Heated...	2	2	2	2
Platanus..Control..	1	1	1	1
Platanus..Heated...	1	1	1	1

TABLE XXXI.

EFFECT OF LONG-CONTINUED HEATING ON THE AB AND MN REACTIONS.

	Precipitin reaction with:			
	Ribes		N.Rusbyi	
	Control	Heated	Control	Heated
N.alata..Control	1	1	3	2
N.alata..Heated	1	1	2	1

TABLE XXXII.

APPLICATION OF SILBERSCHMIDT'S METHODS OF PRE-EXTRACTION AND SOLUTION TO THE AB AND MN REACTIONS.

		No pre-extraction:					Pre-ex.in alc.			Pre-ex.in alc.eth.chl.			
Normal extract:	Experim. extract:	H_2O	NaCl	H_2O+ NaCl	NaCl +MgO	NaOH	H_2O	NaCl	NaCl +MgO	H_2O	H_2O+ NaCl	NaCl	NaCl +MgO
(MN reaction)													
N.Rustica	N.Rusbyi	2	2	2	2	(1)	0	0	0	0	0	0	0
N.Rusbyi	N.rustica	2	2	2	2	(1)	1	1	t	t	t?	t	t
(AB reaction)													
N.Rusbyi	Platanus	2	2	2	0	0	1	1	0	t	t	t	0
N.Rusbyi	Ribes	3	3	3	1	(vv)	2	2	0	1	1	2	0
N.rustica	Platanus	1	1	1	0	(1)	t	t	0	t	t	t	0
N.rustica	Ribes	2	2	2	2	(1)	1	1	t	1	t	1	t
Platanus	N.Rusbyi	2	2	2	2	(1)	t	t	1	t	t	t	1
Platanus	N.rustica	2	2	2	2	(2)	t	t	1	t	t	1	1
Ribes	N.Rusbyi	3	3	3	3	(1)	1	2	2	t	2	2	2
Ribes	N.rustica	3	3	3	3	(2)	2	1	2	t	1	1	t

B. Experiments in Extraction and Solution

As an extension of the scope of the experiment reported on page 149 regarding the effect of Silberschmidt's various techniques upon the resultant reactions, this experiment was repeated with the AB and MN reactions. The plan of the experiment was exactly as in the earlier one. Pre-extractions were of three types: (a) none, (b) with 95% alcohol + tartaric acid, and (c) with tartaric alcohol followed by ether, chloroform, and chloroform vapor. Solution was in water, physiological saline, water + solid NaCl in concentration of .85%, physiological NaCl + MgO to neutrality, and N/20 NaOH. The results of this experiment are given in Table XXXII.

The reactions involving NaOH are not considered significant because of the reaction of the controls with a pure solution of this solvent. These reactions thus represent alkalinity coagulations and not true precipitin reactions. The remainder of the reactions are all significant, however.

With regard to principle M, this experiment shows that it is completely soluble or denatured in 95% alcohol under these experimental conditions. N is somewhat affected by 95% alcohol. The use of physiological saline in place of water as the solvent exerts no perceptible effect on the reaction, regardless of whether the NaCl is present after or before extraction. Extracts in water may thus be freely tested against extracts in NaCl without artefact reactions appearing (this point has also been many times confirmed in subsequent tests).

Principle A is moderately soluble or denatured in alcohol and the lipoid solvents. It is soluble preferably in acid solution, not in neutral solution, and it is equally soluble and reactive in water and physiological saline. Principle B, on the other hand, is equally soluble in water and in physiological saline and in acid and neutral solutions; it is soluble or denatured to a moderate extent in alcohol and the lipoid solvents.

Thus this experiment establishes definitely the fact that the AB and MN reactions are not only distinct from the calcium oxalate reaction (which was eliminated in this experiment by the control tests) but that they are distinct from each other. That the A and B principles are definitely soluble or denatureable in alcohol points to their organic nature. This experiment likewise indicates that the techniques used by Silberschmidt may exert a very distinct effect on the strength of the reactions observed. Some of the inconsistencies which he was unable to account for undoubtedly are to be explained in terms of the solubilities of the reactive substances, and a subsequent part of this paper will

accordingly be devoted to a consideration of his results in the light of this and other experiments.

The question of alcohol solubility thus appears to be a very important one, and accordingly a second, more thorough experiment concentrating on this factor was devised. Leaves of *Platanus* (A), *Ribes* (A), *Nicotiana tomentosa* (B, N), and *N. Rusbyi* (M) were dried and pulverized; 1 gm. of each was extracted in physiological saline for 2 hrs. at room temperature. One gm. of each was pre-extracted in *many*

TABLE XXXIII.

EFFECT OF THOROUGH PRE-EXTRACTION WITH ALCOHOL AND ETHER ON THE AB AND MN REACTIONS.

Effect on principle M:

Rusbyi	a	+	tomentosa	a...2	Ribes	a	+	tomentosa	a...1	Platanus	a	+	tomentosa	a...1
"	b	+	"	a...0	"	b	+	"	a...1	"	b	+	"	a...1
"	c	+	"	a...2	"	c	+	"	a...1	"	c	+	"	a...1
"	d	+	"	a...0	"	d	+	"	a...0	"	d	+	"	a...0
"	e	+	"	a...2	"	e	+	"	a...t	"	e	+	"	a...t

Effect on principle N:

Rusbyi	a	+	tomentosa	a...2	Ribes	a	+	tomentosa	a...1	Platanus	a	+	tomentosa	a...1
"	a	+	"	b...0	"	a	+	"	b...t	"	a	+	"	b...t
"	a	+	"	c...2	"	a	+	"	c...t	"	a	+	"	c...t
"	a	+	"	d...0	"	a	+	"	d...0	"	a	+	"	d...0
"	a	+	"	e...2	"	a	+	"	e...1	"	a	+	"	e...1

Effect on M and N:

Rusbyi	a	+	tomentosa	a...2	Ribes	a	+	tomentosa	a...1	Platanus	a	+	tomentosa	a...1
"	b	+	"	b...0	"	b	+	"	b...1	"	b	+	"	b...t
"	c	+	"	c...2	"	c	+	"	c...1	"	c	+	"	c...t
"	d	+	"	d...0	"	d	+	"	d...0	"	d	+	"	d...0
"	e	+	"	e...1	"	e	+	"	e...1	"	e	+	"	e...t

Effect on principle A:

Ribes	a	+	tomentosa	a...1	Platanus	a	+	tomentosa	a...1					
"	b	+	"	a...1	"	b	+	"	a...1					
"	c	+	"	a...1	"	c	+	"	a...1					
"	d	+	"	a...0	"	d	+	"	a...0					
"	e	+	"	a...t	"	e	+	"	a...t					

Effect on principle B:

Ribes	a	+	tomentosa	a...1	Platanus	a	+	tomentosa	a...1					
"	a	+	"	b...t	"	a	+	"	b...t					
"	a	+	"	c...t	"	a	+	"	c...t					
"	a	+	"	d...0	"	a	+	"	d...0					
"	a	+	"	e...1	"	a	+	"	e...1					

Effect on principles A and B simultaneously:

Ribes	a	+	tomentosa	a...1	Platanus	a	+	tomentosa	a...1					
"	b	+	"	b...1	"	b	+	"	b...t					
"	c	+	"	c...1	"	c	+	"	c...t					
"	d	+	"	d...0	"	d	+	"	d...0					
"	e	+	"	e...1	"	e	+	"	e...t					

changes of absolute alcohol; 1 gm. of each was pre-extracted in many changes of anhydrous ether. There were about 9 changes of pre-extractant in each case, each change lasting 2 hours. The pre-extracted supernatants were evaporated down and redissolved in physiological saline for testing. The pre-extracted residues were dried and extracted in physiological saline. Thus for each species the following 5 extracts were available:

a. No pre-extraction, whole extract (control) in physiological saline.
b. Alcohol extractive in physiological saline.
c. Alcohol residue in physiological saline.
d. Ether extractive in physiological saline.
e. Ether residue in physiological saline.

These were tested against each other according to the scheme of Table XXXIII.

From a consideration of this table one may conclude that under these more thorough experimental conditions:

1. Principles A, B, M, and N are all insoluble in ether and unaltered by ether treatment. They are hence non-lipoid.
2. Principles A, B, M, and N are all non-coagulable by alcohol, even when treated for 24 hours with absolute alcohol at 50°C.
3. *Principles M and N are wholly insoluble in absolute alcohol.*
4. *Principles A and B are both very appreciably soluble in absolute alcohol.*
5. No difference was seen in the behavior of A from that of B nor in M from that of N.

The results of these two experiments are summarized in the following table for the reader's convenience (Table XXXIV).

TABLE XXXIV.

SOLUBILITY AND REACTIVITY OF PRINCIPLES A, B, M, AND N IN VARIOUS SOLVENTS.

Reactive principle:	Solubility in:		Reactivity in:				
	Alcohol	Ether	Water	Water + NaCl	.85% NaCl	NaCl + MgO	NaOH
calcium oxalate	None	None	Good	Good	Good	Good	Results not significant because of artefact.
"A"	Moderate	None	Good	Good	Good	Poor	
"B"	Moderate	None	Good	Good	Good	Good	
"M"	None	None	Good	Good	Good	Good	
"N"	None	None	Good	Good	Good	Good	

C. Experiments in Dialysis

Dialyses of the extracts to determine the diffusability of the AB and MN principles were performed in the same fashion as with *Platanus*, *Prunus*, and *Robinia* as described on page 135. No further comment is necessary beyond the statement that the dialyses of the *Solanaceae* were usually performed using physiological saline rather than distilled water as the fluid outside the membrane. This was desirable practically because it is difficult to obtain clear extracts of *Nicotiana Rusbyi* and certain other *Solanaceae* in water, and theoretically because the protein which might hypothetically be responsible for these reactions might be precipitated by removal of the electrolytes. The experiments of the previous section, however, have revealed that there is no choice in the use of these two solvents as far as demonstrating the precipitin tests is concerned, and furthermore in all experiments here and elsewhere where both aqueous and saline extracts were used in the same experiment,

control tests against the respective solvents confirmed the absence of artefact reactions due to choice of solvent. Since the $AgNO_3$ test for freedom from chloride could not be used in dialyses in NaCl, the oxalate-calcium test, of comparable sensitivity, was substituted where necessary. The results of the dialysis experiments are gathered in Table XXXV.

Considering first the dialyses of *Platanus* (A) one observes that the

TABLE XXXV.

EFFECT OF DIALYSIS ON THE REACTIVE PRINCIPLES OF REACTIONS AB AND MN.

A. Effect on principle A

Extract dialyzed:	Nature of extract:	Exp. #:	Dialysis tests: Prot.	Cl.	Ca.	Sugar	Precipitin tests of experimental extract against: Extract:	Test:	Extract:	Test:	Extract:	Test:
Platanus	Normal	5-10	4	4	3		N.tomentosa	3	N.glauca	3		
"	Dialyz.	8	2	t	t		"	2	"	1		
"	"	9	3	t	t		"	2	"	2		
"	"	7	2	2	t		"	1	"	2		
"	"	5	2	t	t		"	1	"	2		
"	"	10	3	1	t		"	2	"	1		
"	Diffus.	8	0	4	1		"	0	"	0		
"	"	9	t	4	3		"	0	"	0		
"	"	7	0	4	3		"	0	"	0		
"	"	5	0	4	3		"	0	"	0		
Robinia	Normal	19-26	4	4	4		"	3	"	3		
"	Dialyz.	24	3	1	t		"	3	"	2		
"	"	20	2	1	t		"	2	"	2		
"	"	25	4	2	t		"	3	"	3		
"	"	23	4	1	t		"	2	"	2		
"	"	22	2	1	t		"	2	"	2		
"	"	26	4	2	1		"	2	"	3		
"	"	21	4	2	0		"	2	"	3		
"	"	19	3	1	t		"	3	"	2		
"	Diffus.	19	2	4	2		"	2	"	1		
"	"	21	1	4	2		"	1	"	2		
"	"	22	2	4	2		"	1	"	2		
"	"	23	2	4	2		"	2	"	2		
"	"	20	4	3	1		"	2	"	3		
"	"	24	2	4	2		"	2	"	3		
"	"	25	1	4	2		"	1	"	2		

B. Effect on principle B

N.tabacum	Normal	27	3	4	3				Platanus	1		
N.tomentosa	"	34	4		3	4	Robinia	2	"	1		
"	"	35	4		4	4	"	2	"	1		
N.rustica	"	37	2		4	4			"	2	Ribes	4
N.alata	"	38	2		4	3	"	2	"	1	"	2
"	"	39	2		3	3	"	2	"	1	"	2
"	"	40	4		3	2	"	2	"	1	"	2
"	"	41	4		4	2	"	2	"	1	"	2
"	"	42	2		4	4					"	1
"	"	43	2		4	4					"	1
"	"	44	2		3	4					"	2
"	"	45	2		3	4					"	2
N.tabacum	Dialyz.	27	2	1					"	t		
N.tomentosa	"	34	3		t	0	"	1	"	1		
"	"	35	3		t	0	"	1	"	1		
N.rustica	"	37	1		0	0			"	1	"	2

TABLE XXXV. (Continued).

Extract dialyzed:	Nature of extract:	Exp. #	Dialysis tests: Prot.	Cl.	Ca.	Sugar	Precipitin tests of experimental extract against: Extract:	Test:	Extract:	Test:	Extract:	Test:
N.alata	Dialyz.	38	1		t	0	Robinia	1	Platanus	0	Ribes	1
"	"	39	1		t	0	"	1	"	0	"	1
"	"	40	2		0	0	"	1	"	0	"	1
"	"	41	2		0	0	"	1	"	0	"	1
"	"	42	2		0	t					"	1
"	"	43	2		0	t					"	1
"	"	44	1		t	t					"	2
"	"	45	1		t	t					"	2
N.tabacum	Diffus.	27	0	4					"	1		
N.alata	"	44	t		3						"	0
"	"	45	t		3						"	0
C. Effect on principle M												
N.Rusbyi	Normal	28	4		4				N.tabacum	1	N.rustica	3
"	"	29	4		4				"	1	"	3
"	"	33	3		3	4	N.nudicaulis	1			"	2
"	"	34	3		3	4	"	1			"	2
"	Dialyz.	28	4		0				"	1	"	2
"	"	29	4		0				"	1	"	2
"	"	33	3		0	0	"	1			"	2
"	"	34	3		0	0	"	1			"	2
D. Effect on principle N.												
N.alata	Normal	38	2		4	3	N.Rusbyi	2				
"	"	39	2		4	3	"	2				
"	"	40	4		4	2	"	3				
"	"	41	4		4	2	"	3				
"	"	42	2		4	4	"	3				
"	"	43	2		4	4	"	3				
"	"	44	2		3	4	"	2				
"	"	45	2		3	4	"	2				
"	Dialyz.	38	1		t	0	"	0				
"	"	39	2		t	0	"	0				
"	"	40	2		0	0	"	0				
"	"	41	2		0	0	"	0				
"	"	42	2		0	t	"	0				
"	"	43	2		0	t	"	0				
"	"	44	2		t	t	"	0				
"	"	45	2		t	t	"	0				
"	Diffus.	44	t		3		"	2				
"	"	45	t		3		"	1				

Notes: Prot.: Millon test for ptotein; Cl.: $AgNO_3$ test for chloride; Ca.: potassium oxalate test for calcium; Sugar: Fehling test. Extracts in right hand columns all normal. Dialyz.: Dialyzate; Diffus.: Diffusate.

technique of dialysis was satisfactory as is evinced by the dialysis tests. The fractionation resulted in a retention of most of the Millon protein and very little of the crystalloid content. The reactive principle A was retained in amount comparable to the retained protein in the dialyzates. The diffusates, protein-free and containing almost all of the crystalloids, were absolutely negative to the precipitin tests. The results with *Robinia* are less clear cut, but still significant. The dialyzates contained the bulk of the protein and an appreciable but minor amount of crystalloids. The bulk of the salts had plainly passed through to the diffusates, although some protein had also escaped. The results may be

considered safe by comparison, however, the precipitin reaction as in *Platanus* being strongest in the high-protein, low-crystalloid fraction. We may thus conclude from these dialyses that *principle A diffuses very slowly through membranes relatively impermeable to protein.*

Turning now to principle B, the dialyses were technically highly efficient. Most of the protein was reclaimable in the dialyzates, but the latter were practically or entirely negative for calcium and sugar. However, with this highly efficient fractionation, nevertheless a very appreciable amount of the precipitin reaction was lost in dialysis. This is true of the various species employed in these B dialyses all of which behaved similarly. It is thus apparent that, like principle A, *principle B diffuses, although imperfectly and slowly, through membranes highly impermeable to protein but highly permeable to crystalloids.*

The results with the M and N principles are beautifully clear-cut and striking. With regard to principle M in *Nicotiana Rusbyi* the dialyzates retained *all* the protein and *none* of the calcium or sugar according to the dialysis tests. Moreover the precipitin reaction was retained in its full strength in the dialyzates. On the other hand, although the dialyses were almost equally sharp and clear-cut with regard to principle N, the latter was completely lost from the dialyzates in company with the crystalloid content, although very little protein passed through the membranes. We may hence conclude that *principle M is retained by protein-impermeable membranes with a high degree of efficiency, while principle N readily passes through such membranes.*

D. Nature of the AB, MN, and XY Reactions

The experiments in dialysis conclude our investigation of the nature of the AB and MN reactions as far as it has been accomplished at the present time. However, although it is impossible at the present definitely to ascribe these reactions to specific substances, yet from the data obtained one can eliminate many possibilities and arrive at an approximate idea of the nature of the reactive substances involved. We are now able to utilize the following facts which have been demonstrated above.

Principles A and B are water soluble, alcohol soluble, coctostable, insoluble in lipoid solvents, not coagulated by alcohol, and slowly and imperfectly dialyzable through membranes relatively impermeable to protein. The precipitate resulting from their interaction chars markedly on ignition. It is thus plain that they are organic, non-lipoid, and non-protein (because of their alcohol solubility and positive, although imperfect, dialysis). The only alcohol-soluble proteins, the prolamines,

are insoluble in water (19). Thus far no satisfactory distinction has been found between A and B.

Principles M and N are likewise soluble in water and weak salt solutions, but are insoluble in alcohol, non-coagulable by alcohol, insoluble in lipoid solvents, and in contrast to A and B are thermolabile. The precipitate formed by their interaction chars on ignition. M is retained by dialyzing membranes with high efficiency, but N passes freely through such membranes. Thus we may conclude that M and N are both organic and non-lipoid. N is in all probability an organic substance of relatively simple structure, and in any case it is highly unlikely that it is protein. M, on the other hand, gives strong indications of being a protein or at least a complex organic substance of high molecular weight.

With regard to the XY reaction, nothing can be said as to its nature at the present. This is the least frequent, and because of its weakness and rarity the least significant of the four reactions studied. It is plain from Table XXVIII that the species of *Datura, Physalis,* and *Atropa* are alike in possessing a component X which reacts with *Ligustrum* (Y). Whether or not the reactions of *Solanum Capsicastrum* against the *Oleaceae* are qualitatively the same or different is questionable. Because of the weakness and infrequency of this reaction or group o reactions, no analytical tests have been employed concerning it.

VII. THEORETICAL AND PRACTICAL SIGNIFICANCE OF THIS STUDY

In the light of the investigations reported in this paper, it is now of importance briefly to review the results obtained by the various investigators in the field of the plant precipitin reactions for the purposes of culling out the interpretations now known to be erroneous, of supporting with experimental proof those which are correct, of evaluating the immunological significance of the plant precipitin reactions, and of thus placing the studies regarding such reactions upon a solid experimentally-determined foundation. Accordingly the present section will be devoted to these more important theoretical and practical considerations.

A. Analysis of Kostoff's Results in the Light of the Present Study

Kostoff's studies on the precipitin reaction in plants (12) were restricted to 20 species and a hybrid of the *Solanaceae*. The materials on which the findings of the present study are based included 16 of the 20 species on which Kostoff worked. Of the 157 *normal* precipitin reactions of these 20 species tabulated by Kostoff we have repeated all

but 52, with the following results (compiled from Kostoff's Table 10, l. c. p. 49, and from our Tables XXV and XXVI):

Negative reactions reported by Kostoff and confirmed by us 64
Positive reactions reported by Kostoff and confirmed by us 33
Due to calcium oxalate 23
Due to the MN reaction 10
Reactions (positive and negative) reported by Kostoff and not confirmed by us 53
Positive reactions reported by Kostoff and found by us to be negative 7

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Note: With regard to *Capsicum*, Kostoff used *C. pyramidale* while we used *C. frutescens*, but the reactions of these two species are clearly the same, judging from his and our data. The other species involved were identical and largely from the same stock.

Of the 27 *acquired* precipitin reactions described by Kostoff in the *Solanaceae* we have repeated the normal reactions in 10 of the combinations with the following findings:

Marked acquirement of precipitins according to Kostoff 5
Found by us to be due to calcium oxalate 3
Found by us to be due to the MN reaction 1
Not tested by us 1
Slight acquirement of precipitins according to Kostoff 7
Found by us to be due to calcium oxalate 0
Found by us to be due to the MN reaction 2
Found by us to be negative 1
Not tested by us 4
No acquirement of precipitins according to Kostoff 11
Found by us to be due to calcium oxalate 1
Found by us to be negative 9
Not tested by us 1
Decrease of precipitins according to Kostoff 4
Found by us to be due to the MN reaction 3
Not tested by us 1

We thus see that of the 33 normal precipitin reactions reported by Kostoff and repeated by us, 70% were due to calcium oxalate and 30% to the MN reaction. It is of interest to compare these results with those obtained by us in all our normal precipitin tests. The total number of combinations tested by us was 869. Of these, 444 were negative and 425 positive. Of the 425 positive tests 242, or 57%,

were due to the calcium oxalate reaction, 100, or 24%, were due to the AB combination, 73, or 17% were due to the MN reaction, and 10, or 2+% were due to the XY pair.

Thus it is evident that in so far as they have been repeated, Kostoff's positive reactions were all due to the calcium oxalate and MN reactions, mainly to the former. Since the calcium oxalate reaction is inorganic and more or less incidental to the autonomy of the plant, it is manifest that no immunological conclusions can be drawn from an observation of such reactions. Moreover, with regard to the MN reaction, since one reactive group (N) at least is plainly non-protein, and since in the majority of cases where Kostoff dealt with this reaction he observed no change or even decrease, rather than increase, in precipitin reaction after grafting, no sound immunological conclusions may be drawn from this reaction. Kostoff's experiments in nephelometric determination of precipitins (13, 14, 15, 15a) are subject to the same criticism as his gross precipitin tests.

Kostoff's experiments *in vitro* include a discussion of "lytic rings" at the line of junction of the two extracts. These, he believes, are comparable to lytic reactions in animals. In later papers (14, 15, 15a) Kostoff has reported a confirmation of the reactions believed by him to be lytic, by means of dialysis-Ninhydrin tests. Regarding these last tests the authors have nothing to say, since they have not yet been repeated. But with regard to Kostoff's "lytic rings" these have been frequently observed and studied. In his first paper on this subject (3) the senior writer mentioned having observed them in his oleaceous tests but he found them to be "not consistent, weak, and apparently of no greater significance." He was unable, however, to demonstrate lysins in the oleaceous extracts in preliminary experiments using the Ninhydrin method.

That the "lytic ring" phenomenon is not immunological in nature is evident from the following observation transcribed from our notes on one of the preceding experiments. "On May 4th as a control of Experiment 83 the *Ribes Carrierei* extract (N/2 in physiological saline) was layered with N/100 potassium oxalate. The ring resulting (calcium oxalate) was about +1 in strength, clear, and showed the clearest, most striking double ring separated by a clear zone which I have ever seen" (Chester). This was confirmed by two other observers, and was undoubtedly Kostoff's "lytic ring," but occurring in the layering of an extract with an inorganic salt! The same was repeated several times the following day with similar results in all cases. It was not a function of any particular way of pipetting, but careful layering was necessary to bring it out.

It is therefore evident that Kostoff's precipitin reactions and his "lytic rings" are both of the same types as the corresponding phenomena studied by the writers, which are assuredly non-immunological in nature, and that accordingly, on the basis of the data published thus far by Kostoff, one is not justified in drawing immunological conclusions.

B. Analysis of Silberschmidt's Results in the Light of the Present Study[1]

Silberschmidt's experiments (21) dealt with the following species of *Solanaceae*: *Nicotiana Tabacum, N. rustica, N. glutinosa, Solanum Dulcamara, Lycium barbarum,* and *Salpiglossis sinuata.* According to our findings these species had the following complements of reactive principles. The three species of *Nicotiana* all contained an excess of calcium and of principle N; *Salpiglossis* contained excess oxalate and lacked both M and N. From the behavior of Silberschmidt's other two species, *Solanum Dulcamara* probably contained a weak excess of calcium and principle N, while *Lycium* probably contained a slight excess of oxalate and lacked M. The calcium ion was evidently strongest in Silberschmidt's *N. Tabacum* and *N. rustica* and rather weak in *S. Dulcamara* and *N. glutinosa,* while the oxalate was evidently stronger in *Salpiglossis* than in *Lycium,* but not very strong in either.

Calcium oxalate reactions are plainly evident from Silberschmidt's tables (e.g. *Salpiglossis* + *N. rustica* and *N. Tabacum* in his Table 2). The clearness of his readings was at times obscured by the cloudiness and dilution of his extracts. This applies particularly to his data in Tables 3, 5, and 6. In his Tables 9, 10, 11, and 12 the reactions are all negative or practically so as would be expected from such reactive complements as those outlined above. The only reactions requiring comment are two involving *N. Tabacum.* In Silberschmidt's Tables 3 and 4 *N. Tabacum* reacts positively with *Solanum Dulcamara* and with *N. rustica.* This *N. Tabacum* reaction behaves much as the MN, and it would be reasonably explained on the assumption that *N. Tabacum* (which is closely related to *N. Rusbyi,* the type of principle M), sometimes contains a sufficient quantity of M to react with active N extracts. In accordance with this view is the fact that Kostoff's *N. Tabacum* reacted, although weakly, with two of the other *Nicotiana* species used. As was mentioned earlier in this paper the presence of M in *N. Rusbyi* varies considerably from plant to plant and a corresponding variability

[1] A second paper by this author has recently been published. This paper [Silberschmidt, K. (1932). Studien zum Nachweis von Antikörpern in Pflanzen. II. (Planta: Arch. f. Wiss. Bot. 17:493-589)] is subject to the same criticism as the paper under discussion.

in *N. Tabacum* would perfectly account for the apparent discrepancies between Silberschmidt's results and those of Kostoff and the present writers.

Such an analysis accounts for such of Silberschmidt's reactions as are significant, while his negative results in the few places where one would expect positive reactions are apparently due either to lack or to weakness of reactive principles in the various combinations. His one acquired reaction is hardly of strength to be considered significant.

Hence one may conclude, with regard to Silberschmidt's work, that it is confirmed in fact, but not in theory, by the present study. For the same criticisms regarding immunological interpretations as were made of Kostoff's results are applicable to those of Silberschmidt. His positive reactions are all explainable in the terms of the relatively simple reactions described in this paper, and there is no evidence that any of them are due to protein interaction.

C. Analysis of East's Results in the Light of the Present Study

East (7) has observed certain phenomena *in vivo* with regard to recovery of sugar cane from the mosaic disease and to temporary resistance to re-infection. In connection with these observations he carried out a series of precipitin tests in order to try to decide whether or not the proteins of various types of cane were different. His experimentation followed the same general type as that used by Kostoff and Chester, and accordingly his results may be considered comparable. In certain combinations of extracts of cane with those of cane and other plants East obtained positive results. He found some indication of a consistent difference in precipitin reaction between cane which had never had the mosaic disease and cane which had or may have had the disease. Since plants which exhibited the mosaic disease reacted in the same manner as plants which appeared free from disease but which might have been acting as carriers, and since the reactions of such plants consistently differed from those of cane known never to have had the disease, East believed that his results, including his observations on infection and recovery, pointed to the probability that sugar cane gains an apparent immunity by reducing the virulence of the mosaic virus.

The results of the present study render it very questionable as to whether the precipitin experiments with sugar cane should be considered immunological. Since the writers have not experimented with the species of plants used by East, it is impossible dogmatically to relegate his reactions to the same category as the non-protein reactions found to occur so extensively in the *Solanaceae* et al., but assuredly one

must be rather skeptical in accepting as immunological any precipitin experiments in which the possibilities of such non-protein reactions as the calcium oxalate and AB reactions are not eliminated.

D. Analysis of Chester's Results in the Light of the Present Study

The findings of Chester with regard to the precipitin reaction (3, 4, 5) have not been definitely interpreted as immunological, pending the results of the present study. Chester's findings prior to this study may be stated in the following terms:

1. No normal precipitins were found in the *Oleaceae* studied.
2. Graft-blighted Lilacs showing serious malnutritional symptoms gave strong positive tests with other *Oleaceae* regardless of systematic relationship to stock or scion. Lilacs suffering from similar symptoms but ungrafted gave the same reactions.
3. In tests in a number of families of woody plants a clear-cut parallel was observed between the precipitin reaction and the systematic relationships of the plants tested.

It is the purpose of the present section to criticize and interpret these findings.

The tests reported in this paper have shown that in every case tested, the *Oleaceae* were characterized by having an excess of calcium present in their extracts. In the morbid processes attending the slow dying of leaves of Lilac there is an accumulation of oxalate. This has been shown by the fact that reactive extracts of graft-blighted plants may be rendered inactive toward "calcium" extracts by precipitating them with a slight excess of calcium. Such oxalate accumulation is localized in the diseased and dying cells, since when the green portions of green-and-yellow mottled leaves are cut away from the yellow portions and extracted, the extracts of these green portions behave exactly like extracts of healthy Lilacs. This fact renders it still more improbable that the reactions of graft-blighted Lilacs are immunological, since hypothetical antibodies from grafting would be expected to be generally distributed through the leaf tissue. The fact that leaves of plants mechanically injured are also reactive in the yellow areas supports this simple interpretation, as does the fact that extracts of mottled leaves autoprecipitate in extraction, losing their reactive potency as autoprecipitation continues, due to the interaction of the excess oxalate in the yellow portions with the excess calcium in the green portions.

Passing next to the highly absorbing topic of the specificity of the normal precipitin reactions thus far investigated, the results of this

study are of especial interest. *A priori* it was difficult to conceive that such specificity as was seen in the *Prunoideae* and the other groups of woody plants studied could be due merely to the interaction of relatively non-specific substances. However the distribution of many chemical substances in plants follows in general their relationships. Thus the presence and form of calcium oxalate crystals in plants have been frequently used as taxonomic criteria, and it is manifest from a study of the data on distribution of other substances, such as saponins, glucosides, alkaloids, etc., that homogeneous groups of plants are characterized by homogeneity in their content of such substances.

Guided by such reasoning it was of interest to examine the distribution of the various reactive principles differentiated in this study to observe whether they showed any species-, genus-, or family-specificity in their distribution among the various plants studied. From the results of Tables XXV-XXVIII it was possible to ascribe to each species its "reactive formula." This, for example, with *Salpiglossis sinuata* was: A—B+, M—N—, X—Y—, Ox+Ca—. These were formulated for each of the species studied and then they were grouped in a trifurcating dendritic system as in Figure IV. The order of arrangement was guided by the observation that certain of the reactions varied within the genus, others within the family, etc.

A survey of Figure IV brings out some very interesting relationships. The five families used in this study fall into three orders, the *Rosiflorae*, including the closely related *Platanaceae*, *Saxifragaceae*, and *Leguminosae* (which three are adjacent in Rehder's Manual), the *Contortae*, including the *Oleaceae*, and the *Tubiflorae*, represented by the *Solanaceae*. With but one questionable exception the *Rosiflorae* studied all possess A and lack B, the *Tubiflorae* (*Solanaceae*) all contain B and lack A, while the *Contortae* (*Oleaceae*) all lack both A and B. *Prunus* lies very near the borderline, and might without difficulty be included in either group. Within the *Rosiflorae* subsequent division was not attempted because of the difficulty of eliminating the AB reaction in considering the MN and XY reactions. Within the *Oleaceae* all contain excess of Y and of calcium and are separated only by presence or absence of M and N, a highly homogeneous assortment of reactive principles as would be expected from their relative taxonomic homogeneity.

Within the *Solanaceae* none contain the principle Y (which was found in the *Oleaceae*) and the majority also lack X, although this is present in a few species. The *Solanaceae* are differentiated by both the MN and calcium oxalate reactions, the latter, however, being con-

sidered, because of its simple nature, as least significant. Of the 18 species of *Nicotiana* all are identical in their reactive ingredients with the sole exception of *N. Rusbyi*. *Datura* varies with regard to the XY reaction but is relatively uniform with regard to the presence of N and

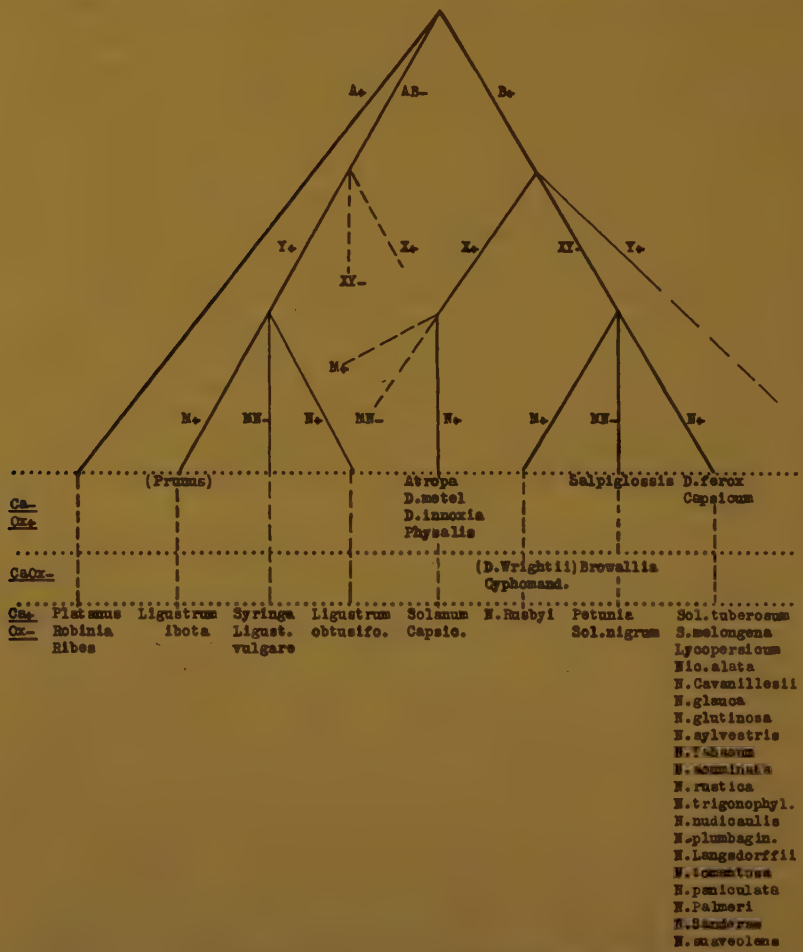

FIGURE IV. CLASSIFICATION OF ALL EXTRACTS STUDIED ACCORDING TO THE PRESENCE OR ABSENCE OF THE VARIOUS REACTIVE PRINCIPLES.

of excess oxalate. The *Solanum* species are all uniform in their excess of calcium, although there was some individual variation of this in *S. tuberosum*, while they are separated by the presence or absence of MN and X.

Taken on the whole, there seems to be a definite tendency for the various taxonomic units to show relatively homogeneous reactive formulae. There are exceptions, to be sure, but these exceptions are by no means as striking as the agreements. That such a dendritic system as that of Figure IV could result from chance is beyond belief. It is entirely possible that an extension of these results to include many more species might either favorably or unfavorably influence this relationship, but the data presented appear to justify, at the present, the statement that there is a definite indication of a correlation between the distribution of reactive principles in the plants studied and their taxonomic position.

Hence the results obtained by the senior writer on the *Rosaceae, Saxifragaceae, et al.* are interpreted by the results of the present study. If there is even a moderate and imperfect degree of correlation between the systematics of these plants and their complement of reactive principles, then it necessarily follows that this correlation will be reflected in a corresponding correlation of precipitin reaction with systematics.

Before, however, drawing final conclusions regarding the specificity of the normal precipitin reaction in plants it is well to consider the variability of the reaction among individuals of a given species. Thus far none of the published accounts have been concerned with the reactions of more than one or a few individuals of a given species. Kostoff does not record the number of repetitions of each normal test. Silberschmidt's data on the subject are so limited as not to afford significant comparative data. Chester's experiments with the woody plants apart from the *Oleaceae* were based on one or very few extracts for each species, and his results with the *Oleaceae* were the only ones published in which a generous assortment of individuals of each species was used. In the latter case, however, although his results show the uniformity of the various individuals and varieties of a species, the fact that the basic reaction here is negative weakens the force of the argument regarding the constancy of the precipitin reactions in a species.

In order to test this matter an experiment was performed by the writers utilizing numerous individuals of a few selected species but under different experimental conditions. Three questions were investigated, namely: What is the effect of age of plant on the precipitin reactions? What is the effect of the addition of calcium and oxalate to the soil upon the precipitin reactions? What variability is there between different horticultural varieties and individuals of a given species? For this experiment cultivated Tomato (*Lycopersicum esculentum*...4 varieties), Pepper (*Capsicum frutescens*...2 varieties), and

Potato (*Solanum tuberosum*...2 varieties) were employed. In the testing of varietal differences all 8 varieties were used, likewise all the varieties were used in testing the effect of addition of salts to the soil, while for the tests on age variations the tomato variety *Bonny Best* was employed. Five or six individuals of each variety were used in each part of the experiment. The experiment on age differences consisted in weekly testing of 5 plants of the tomato variety from the time they were 6-8″ in height to maturity. That on the effect of fertilization consisted in testing the 48 plants used after having treated one third of them with lime, one third with daily applications of 1:1000 potassium oxalate, and leaving one third untreated as controls. The treatments lasted for one month. The tests which were then performed in every case comprised tests with a N/100 potassium oxalate solution, to determine the calcium oxalate reaction, with *Ribes* to determine the effect on the AB reaction, and with *Nicotiana Rusbyi* to observe variations in the MN reaction. The same tubes of *Ribes* and *Nicotiana* were used in all the tests. Unfortunately the *N. Rusbyi* plant selected at the beginning of the experiment, and therefore of necessity employed throughout the whole of the experiment, was negative for the MN reaction. However, the consistency of its negative reactions, under the various experimental conditions is of significance, even though this significance is not as great as though it had been positive. The results of this experiment are gathered in Table XXXVI.

Let us consider first the question of variation among the horticultural varieties of a given species. A comparison of the reactions, taken as a whole, of the four tomato varieties shows them to be entirely uniform with reference to each of the reactions. The average reactivity of the four tomato varieties to oxalate was 1.4, 1.4, 1.2, and 1.8 respectively; that of the four tomato varieties to *Ribes* was 2.0, 1.4, 2.0, and 2.2 respectively; that of the four varieties toward *Nicotiana Rusbyi* was negative in every case. The same reasoning may be applied to a comparison of the two potato varieties with each other and of the two pepper varieties with each other. In addition to these data, the findings of Chester on the reactivity of 18 horticultural varieties of *Syringa vulgaris* may be cited, in which it was shown that when tested under comparable conditions there was no significant variability of reaction among the varieties employed. We are thus free to conclude that all the evidence thus far obtained indicates that the reactions of any selected horticultural variety of a species are approximately equivalent to those of any other selected variety of the same species.

Passing next to the question of variation among individuals of a given

TABLE XXXVI.

A. VARIABILITY OF THE CALCIUM OXALATE, AB AND MN REACTIONS SHOWING EFFECTS OF INDIVIDUAL AND VARIETAL VARIABILITY AND OF SOIL TREATMENTS.

Tomato variety:	Plant #	Treatment:	Precipitin reactions Before treatment: Ox	AB	MN	After treatment: Ox	AB	MN
Pritchard	1	Lime	1	2	0	2	2	0
"	2	Lime	1	2	0	1	2	0
"	3	Oxal.	2	2	0	1	2	0
"	4	Oxal.	2	2	0	2	2	0
"	5	None	1	2	0	1	2	0
Break O' day	1	Lime	1	2	0	2	2	0
"	2	Lime	1	1	0	1	2	0
"	3	Oxal.	1	1	0	1	2	0
"	4	Oxal.	2	1	0	1	2	0
"	5	None	2	2	0	1	2	0
Norton	1	Lime	1	2	0	1	2	0
"	2	Lime	1	2	0	2	2	0
"	3	Oxal.	2	2	0	2	2	0
"	4	Oxal.	1	2	0	2	2	0
"	5	None	1	2	0	2	2	0
Bonny Best	1	Lime	1	3	0	1	2	0
"	2	Lime	2	2	0	1	2	0
"	3	Oxal.	3	2	0	1	2	0
"	4	Oxal.	1	2	0	1	2	0
"	5	None	2	2	0	1	2	0

Potato variety:	Plant #	Treatment:	Pptin. reaction after treatment Ca	Ox	AB	MN
Green Mt.	1	Lime	0	1	3	0
"	2	Lime	0	t	3	0
"	3	Oxal.	t	0	3	0
"	4	Oxal.	1	0	2	0
"	5	None	0	0	2	0
"	6	None	0	t	3	0
Var.?	1	Lime	0	t	2	0
"	2	Lime	t	0	2	0
"	3	Oxal.	1	0	2	0
"	4	Oxal.	1	0	2	0
"	5	None	0	0	2	0
Pepper var.						
Red Spot of Oshkosh	1	Lime	1	0	2	0
"	2	Lime	1	0	2	0
"	3	Oxal.	1	0	2	0
"	4	Oxal.	1	0	2	0
"	5	None	2	0	1	0
"	6	None	1	0	2	0
Chinese Giant	1	Lime	2	0	2	0
"	2	Lime	1	0	2	0
"	3	Oxal.	1	0	2	0
"	4	Oxal.	1	0	2	0
"	5	None	1	0	2	0
"	6	None	1	0	2	0

TABLE XXXVI.

B. VARIABILITY OF THE CALCIUM OXALATE, AB, AND MN REACTIONS IN TOMATO VARIETY "BONNY BEST," ACCORDING TO AGE OF PLANT.

Plant #:	Date of precipitin tests: May 5 Ox	AB	MN	May 11 Ox	AB	MN	May 18 Ox	AB	MN	May 25 Ox	AB	MN	June 1 Ox	AB	MN	June 8 Ox	AB	MN	June 15 Ox	AB	MN
6	2	3	0				2	2	0				2	2	0				1	3	0
7	2	3	0				2	2	0				1	3	0				1	3	0
8	1	3	0				2	3	0				2	3	0				2	2	0
9	2	3	0				2	1	0				1	2	0				1	3	0
10	2	3	0				2	2	0				1	2	0				1	2	0
11				1	2	0				2	2	0				1	2	0			
12				1	3	0				2	2	0				2	2	0			
13				2	2	0				2	1	0				2	3	0			
14				2	2	0				2	2	0				2	3	0			
15				2	2	0				2	2	0				1	3	0			

variety grown under comparable conditions, we find by consulting Table XXXVI that here too a striking uniformity prevails. The various tests of this table may be divided into 132 pairs, each pair representing a given type of reaction performed on two individuals of the same variety and under comparable experimental conditions. If these 132 pairs of reactions be examined, it will be seen that in 77% of the cases there was no difference in reactivity between the two individual plants of a pair, in 3% of the cases there was only a difference between t and 1, in 19% of the cases there was a difference of one unit between the individuals of a pair, while in only 1% of the pairs was there a difference greater than 1 unit. To these data may be added those of the preceding tables and earlier publications in which it will also be seen that on the whole there is a relatively high degree of agreement between the reactions of individuals of the same species and variety. However, a significant variability has been observed with reference to one of the principles not varied in this table, namely principle M (of *Nicotiana Rusbyi* and *Datura Wrightii*). In our tests as compared with those of Kostoff and Silberschmidt there is evident a certain variability in the occurrence and strength of this reactive principle. M is the least stable of all the reactive groups thus far studied, in conformity with its highly complex nature, and is not a specific character in the same sense as the other reactive principles under observation.

Considering next the variability according to age in the tomato variety Bonny Best, one observes from the table that there is no significant trend of change of reaction strength between the age when testing was first possible (6″ ht.) to the period of maturity. Accordingly we may conclude from the facts at hand that precipitin reactivity is not a variable related to the varying age of the plants tested.

The last question to which this experiment affords an answer is that of the effect of environment on changing the reactivity of individuals. The tomato varieties are all characterized by containing a notable excess of calcium. Treatment of plants of these varieties with lime did not appreciably increase this calcium strength nor did treatment with oxalate appreciably decrease it. The same argument applies to the two varieties of Pepper studied, although in this case the two varieties were characterized by strong excess of oxalate. With regard to the Potatoes, however, a different situation obtains. The potato varieties studied possessed either no excess of calcium and oxalate or only a slight excess of one or the other. They lie on the borderline with respect to the calcium oxalate reaction and accordingly one might expect that here it would be most likely possible to demonstrate an

experimental variation in these principles. Such is the case. With the Potatoes the drastic liming and treatment with oxalate did result in a slight but significant increase of the calcium on the one hand and of the oxalate on the other. We may thus say that the evidence presented justifies the statement that when a species is characterized by a strong calcium or oxalate reaction strong alterations of these elements in the soil fails to produce a demonstrable change in the precipitin reaction, while if a species is characterized by a very weak or negative calcium oxalate reactivity, drastic soil alterations may produce a mild modification of the calcium oxalate reaction. However, such a modification does not seriously disrupt the burden of the significance of the reactions thus far reported since, (a) most species show strong excesses of calcium or oxalate, (b) the soil variation was more drastic than would ordinarily obtain in experimental work on the precipitin reaction, and (c) the differences in reactivity between the controls and the treated plants were not great.

In the light of this experiment we are now able to return to the question of the specificity of the "normal precipitin reaction" and its significance in systematics. We may say that on the whole the experimental data thus far available demonstrate that precipitin reactivity with respect to the various reactive principles studied is a relatively constant species character, that the distribution of the precipitin reactive principles in the various plants tested shows a general agreement with the systematic position of the plants, and that this approximate correlation between presence of reactive principles and taxonomic position is of necessity reflected in a comparable correlation between precipitin reaction and systematics.

As to the practical advantage which may be taken of this correlation, we are now in a better position to judge than before the present analysis was made. It is evident that the precipitin reactions in plants are very different from the precise and specific reactions of animal serology. Hence one cannot consider the two as comparable. One is a protein reaction as far as we know now, the others are far simpler with regard to the nature of the reactive substances but more complex with regard to the number of types of reaction present. The normal precipitin reactions in plants to no extent offer a substitute for the work in the serology of plant proteins. On the other hand, if the taxonomist is interested in studying not only the morphology of his material but also its chemical composition, and recent taxonomic studies have shown the value of such undertakings, then the precipitin technique offers an additional technique of biochemical investigation. It reveals chemical dif-

ferences of varying degrees of significance, although the nature of these differences is not always clear without a chemical analysis, and these differences may have much to offer the investigator of the taxonomy of homogeneous or puzzling plant groups.

E. Immunological Significance of the Precipitin Reactions in Plants

The differences between the precipitin reactions of plants and the reactions of animal serology are so manifest from the preceding experiments that only a brief statement regarding their immunological significance will be introduced at this point.

Of the four reactions which account for all of the interreactions of the 45 species of plants in the present study, no one, so far as we know, is caused by the interaction of protein in one extract with protein in another. The calcium oxalate reaction is a problem in inorganic biochemistry. The AB reaction is due to two interacting substances which, because they are alcohol-soluble, alcohol resistant, and heat resistant, are assuredly non-protein, although they are plainly organic and of large molecular weight. The principle M may be protein in nature but its counter-principle N is relatively simple, apparently crystalloid, and assuredly non-protein. Of the nature of the relatively insignificant XY reaction we know nothing. In the three reactions which we have studied there is no homology with the specific protein reactions of animal serology, in which customarily specific protein reacts with specific protein to produce a precipitation of protein.

It is not inconceivable that other substances than proteins might take part in immunological reactions. Much and Frankel (18) have shown that immunological reactions in animals may be non-specific, Besredka (1) cites instances where they may be localized, and Heidelberger and Avery (10) have shown that immune sera react specifically with a protein-free carbohydrate elaborated by pneumococci. In plants the carbohydrates are much more highly developed than in animals, and it is not impossible that in immunological reactions in plants the carbohydrates would have a much greater rôle than in animals. However, up to the present, the absence of extensive data on this subject requires the utmost caution in formulating theories regarding plant immunological reactions, and the burden of the evidence regarding the nature of the precipitations which have been observed between plant extracts is strongly against any immunological interpretation homologous with that in animal serology. Most assuredly *the work which has been done up to the present on the precipitin reaction in plants does not*

prove that plants can elaborate antibodies as a result of grafting. On the other hand, the *possibility* that plants may be able under certain conditions to elaborate antibodies of the zoöimmunitary type is obviously not eliminated nor is it likely to be with ease because of the relatively small stress which can be laid on negative results in this connection.

F. The Direction of Further Studies in this Field

It is the conviction of the writers that the possibilities in the field of the precipitin reaction in plants are by no means exhausted. Although there is no sound evidence at present demonstrating that, as a result of grafting, plants may acquire or increase their precipitin potency in a zoöimmunitary sense, this thesis is contradicted neither by theory nor by experiment. The absence of normal precipitin reactions in plants does not preclude the possibility of demonstrating acquired reactions, in fact it favors this possibility by eliminating from consideration the artefact non-protein reactions of the types investigated above. It would thus be highly desirable to investigate more graft combinations in this connection, but proceeding in the light of the present study. The number of experiments of this sort which could be performed must necessarily be limited because of the necessity, in passing, of investigating the nature of the reactions found. The biochemical studies reported above will serve as an outline of the procedures found desirable in investigating the nature of such reactions. Certainly no graft reaction should be described as immunological in the zoöimmunitary sense in which the reactive substances are not investigated at least with regard to their solubilities, their behavior in the presence of heat and of strong alcohol, and their relation to dialyzing membranes. In view of the differences manifested by the precipitations in animals and plants an unlimited application of serological terminology to the plant phenomena is to be discouraged. It engenders in the minds of workers in related fields a belief that the reactions in the two fields are homologous. Carbone's term "pseudo-antibodies," although not wholly expressive of the situation, is far preferable to an uncritical use of the terms precipitin, lysin, antigenic extract, etc., and for this reason the writers, here and elsewhere, have attempted to minimize this misconception, where possible, by avoidance or quotation of such terms.

VIII. SUMMARY

The present paper reports an analysis of the nature of the "normal precipitin reaction" in plants. The conclusions reached are based upon more than 4000 precipitin tests, heretofore unpublished, using 45 species

of herbaceous and woody plants as material. Proceeding from an analysis of the reaction between *Prunus, Platanus, Ribes, Robinia,* and *Hydrangea,* the scope of the analysis was extended to include the various interreactions of other woody plants and of 35 of the *Solanaceae.* The results obtained may be summarized in the following points:

1. The interreactive substances of *Prunus, Platanus, Ribes, Robinia,* and *Hydrangea* are characterized by the following properties:
   a. They are coctostable (3 hrs. autoclaving at 0-2 lbs. pressure).
   b. They are relatively insensitive to variations of pH within wide limits. Strong alkalinization or acidification of the extracts does not remove the reactive substances, although the reaction does not occur at very low or very high pH values.
   c. The reactive substance of *Prunus* is not affected by variation of salt concentration within wide limits, that of *Platanus* and *Robinia* steadily decreases in strength as the salt (phosphate) content increases.
   d. On dilution of the extracts there is no evidence of a zone phenomenon such as occurs in animal serology.
   e. The interreactive substances of these plants pass freely through dialyzing membranes highly impermeable to protein.
   f. The interreactive substances in these plants are equally soluble in water and in physiological saline; they are insoluble in strong alcohol, ether, chloroform, carbon tetrachloride, benzol, and 95% alcohol + 1% tartaric acid; they are not denatured by any of these solvents; and the reactions take place with equal facility in the presence of NaCl (.85%), MgO, and N/20 NaOH.
   g. The reactive ingredient in *Prunus* is precipitated by neutral lead acetate but is recoverable in such precipitates, that of the other species is not so precipitable and is reclaimable in the practically carbohydrate-free fraction resulting from neutral and alkaline lead acetate precipitation.
   h. Double precipitation shows that only a single type of reaction is involved between these species.
2. That this reaction is due to the interaction of free oxalate in *Prunus* with free *calcium* in the other extracts has been demonstrated:
   a. By qualitative analysis (the results of which are summarized on page 159).
   b. By quantitative analysis which showed calcium oxalate to be present in the washed precipitates to the extent of 85-90%.
   c. By complete elimination of the reactions by precipitating *Prunus*

with calcium salts or the other extracts with oxalates respectively.

3. This calcium oxalate "precipitin reaction" has been found to occur extensively throughout the woody plants and *Solanaceae* studied. About half of all the reactions obtained in this study were positive, and of these positive reactions 57% were due to calcium oxalate. 70% of our positive tests of the plants used by Kostoff in this work were found to be due to calcium oxalate.
4. By now eliminating the calcium oxalate reaction from consideration it was possible to determine whether other reactions were present among these species. Three such other reactions have been distinguished. One, the AB reaction, is characterized by the reactions of the *Rosiflorae* employed (A) with the *Solanaceae* (B). It accounts for 24% of all the positive tests observed in our material. A second reaction, MN, particularly results from the interaction of *Nicotiana Rusbyi* and *Datura Wrightii* (M) with other *Solanaceae* (N). It is responsible for 17% of our precipitates. The third reaction, XY, occurs between the *Oleaceae* (Y) studied and certain of the *Solanaceae* (X). It is relatively insignificant, being responsible for only 2% of the total reactions observed. The identity and distinctness of these three reactions have been established by various types of experiment.
5. Principles A and B are water-soluble, alcohol-soluble, coctostable, insoluble in lipoid solvents, not coagulable by alcohol, and slowly and imperfectly dialyzable. The precipitate formed from their interaction chars upon ignition. Hence they are plainly non-lipoid and non-protein (because of their alcohol solubility and positive, though imperfect dialysis) but organic and of relatively high molecular weight. Principles M and N are soluble in water and weak salt solutions, but are insoluble in alcohol, are non-coagulable by alcohol, insoluble in lipoid solvents, and in contrast to A and B are thermolabile. The precipitate formed by their interaction chars on ignition. M is retained by dialyzing membranes with high efficiency, but N passes freely through such membranes. Hence they are both organic and non-lipoid. N is in all probability an organic substance of relatively simple structure and it is highly unlikely that it is protein. M, on the other hand, gives strong indications of being protein or at least a complex organic substance of high molecular weight.
6. The published immunological interpretations of precipitin reactions in plants are rendered untenable because of the lack of homology

between the animal and plant reactions, and because of the widespread occurrence in the plants heretofore used of simple, non-protein reactions. The works of Kostoff, Silberschmidt, and East are analyzed in this connection.

7. The findings of Chester with regard to the reactions of diseased Lilacs and to the specificity displayed by the normal reactions are interpreted and substantiated by these findings.
8. The experimental work has shown that the age of the plants does not have a significant effect upon the strength of the calcium oxalate reaction. Treatment of tomato and pepper varieties with lime, and with potassium oxalate did not appreciably alter the strength of the calcium oxalate reaction. The same treatment applied to potato varieties produced a demonstrable change in this reaction. In the later case the slight effect produced by experimental variation of the environment cannot be considered as seriously affecting the significance of the reactions reported.
9. The possibility, in the future, of demonstrating immunological reactions from grafting is not eliminated by this study. There is proof that the reactions thus far reported were doubtless non-immunological in the zoöimmunitary sense, but on the basis of the findings of the present study the desirable directions of future work in this field are pointed out.

IX. ACKNOWLEDGEMENT

The work reported in the present paper has been very appreciably furthered by the assistance and suggestions of a number of workers in related fields. The experiments in chemical analysis of the extracts were materially aided by the coöperation of Drs. Ronald Ferry and Benjamin White. For numerous suggestions and criticisms throughout the study the writers are indebted to Professors J. H. Faull and E. M. East. To them the writers wish, at the present time, to express their thanks.

X. LITERATURE CITED

1. BESREDKA, A. (1927). Local immunization. Plotz translation. (Williams and Wilkins, Baltimore, Md.)
2. CARBONE, D. & C. ARNAUDI. (1930). L'immunità nelle piante. (Monogr. Istituto Sieroterap. Milanese.)
3. CHESTER, K. S. (1931). Graft-blight: A disease of lilac related to the employment of certain understocks in propagation. V. F. Specific physico-chemical or immunological differences between stock and scion. (Jour. Arnold Arbor. **12**:114–128.)
4. ———— (1932). Studies on the precipitin reaction in plants. I. The specificity of the normal precipitin reaction. (Ibid. **13**:52–74.)

5. ———— (1932). Studies on the precipitin reaction in plants. II. Preliminary report on the nature of the "normal precipitin reaction." (Ibid. **13**:285–296.)
6. Cohn, E. J. (1927). The activity coefficients of the ions in certain phosphate solutions. A contribution to the theory of buffer action. (Jour. Amer. Chem. Soc. **49**:173–193.)
7. East, E. M. (1931). Immunity to sugar cane mosaic acquired by the host. (Proc. Nat. Acad. Sci. **17**:331–334.)
8. Fischer, E. & E. Gäumann. (1929). Biologie der pflanzenbewohnenden parasitischen Pilze. (Fischer, Jena.)
9. Gäumann, E. (1928). Das Problem der Immunität im Pflanzenreich. (Vierteljahrsch. Naturforsch. Ges. Zürich. **73**, Beiblatt: Festschrift Hans Schinz, 450–468.)
10. Heidelberger, M. & O. T. Avery (1923). The soluble specific substance of pneumococcus. (Jour. Exper. Med. **38**:73–79.)
11. Kobert, R. (1902). Lehrbuch der Intoxikationen. (Bd. I. p. 161; Bd. II., p. 695 ff. Stuttgart.)
12. Kostoff, D. (1929). Acquired immunity in plants. (Genetics, **14**:37–77.)
13. ———— (1930). Tumors and other malformations on certain Nicotiana hybrids. (Zentralbl. f. Bakt. Abt. 2, **81**:244–260.)
14. ———— (1930). Induced immunity in plants by grafting. (Extrait du Ier Congrès Intern. de Microbiol. Paris, 1930.)
15. ———— (1931). Studies on the acquired immunity in plants. A contribution to the methods for investigating the mutual activities between graft components. (Svedenia po Zemledelieto, **12**:47–69.)
15a. ———— (1931). Studies on the acquired immunity in plants induced by grafting. (Zeit. f. Immunitätsf. **74**:339–346.)
16. Kraus, R. (1902). Zur Theorie der Agglutination. (Zeit. f. Heilk. **23**:369–390.)
17. Mason, V. P. (1922). Relation of pH to specific precipitation. (Johns Hopkins Hosp. Bull. **33**:116.)
18. Much, H. & E. Fraenkel (1911). (*Cited in* Much, H. Tuberkuloseforsch. in Muenchener Med. Wochenschr. **78**(1931):137–139.)
19. Osborne, T. B. (1924). The vegetable proteins. Ed. 2 (Longmans, Green & Co. London.)
20. Rimington, C. (1929). The isolation of a carbohydrate derivative from serum proteins. (Biochem. Jour. **23**:430–443.)
21. Silberschmidt, K. (1931). Studien zum Nachweis von Antikörpern in Pflanzen. I. (Planta: Arch. f. Wiss. Bot. **13**:114–168.)
22. Ward, M. (1905). Recent researches on the parasitism of fungi. (Ann. Bot. **19**:1–54).

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